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There and Back Again: Functional Outcomes of Reciprocal Neuron-Astrocyte Signalling

Alison C. Todd, BSc (Hons)

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University of Edinburgh

Supervisors

Prof. Giles E. Hardingham

Prof. David J. A. Wyllie

Declaration

I declare that this thesis has been composed by myself and that it has not been submitted, in whole or in part, for any other degree or professional qualification. Except where stated otherwise in text, by reference or acknowledgment, the work presented within is my own.

Alison Clare Todd

30 March 2020

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Abstract

Neurons do not exist in isolation in the central nervous system, and there is a growing appreciation that the interactions between neuronal and non-neuronal cells are fundamentally important for nervous system function. A major family of non-neuronal cells are the astrocytes, with a surge of recent work suggesting the relationship between neurons and astrocytes is bidirectional and highly complex. In my thesis I seek to further uncover the nature of this intimate relationship between neurons and astrocytes of the cortex. One well-established role of astrocytes is the collection of neuronal glutamate via their high affinity excitatory amino acid transporters, with dysfunctions in this system being linked to numerous neurological diseases. Previous reports suggest that neurons may regulate the expression of these astrocytic glutamate transporters, through an as yet unknown pathway. In my thesis I first investigate the nature of this non-cell-autonomous neuronal control of astrocytes. I begin by using results from the lab's novel mixed-species RNA-sequencing dataset to explore how neurons regulate astrocytic gene expression, finding that they upregulated the astrocytic glutamate transporters. By electrophysiological recording I show a corresponding functional increase in the astrocytes' ability to collect glutamate, before demonstrating that neurons upregulate the astrocytic transporters through Notch signalling. I then investigate whether continuous Notch signalling is required to maintain these transporters' expression and function, finding that removal of Notch signalling after the establishment of transporter expression significantly reduces the transporters' activity. For the remainder of my thesis I explore how cortical astrocytes may in turn control cortical neuronal function. Using RNA-seq data generated in the lab I discover a host of neuronal genes that are regulated by astrocytes. Amongst these genes were the functionally important K^+ inward rectifying channel family, which were strongly downregulated in neurons by astrocytes, an observation hitherto unseen. I hypothesise that this downregulation will result in alterations to neuronal membrane properties which will enhance neuronal excitability, and that this may in turn have down-stream consequences on neuronal activity and synaptogenesis. I find that cortical neurons are rendered more excitable by astrocytes, leading to an enhancement of neuronal activity, driven by the astrocyte-induced decrease in K^+ inward rectifiers. Although I do not see an increase in baseline synaptogenesis, I show a range of homeostatic neuronal responses emerge in the presence of astrocytes. This work suggests that astrocytes play a central role in regulating neuronal activity.

Lay Summary

There are many different cell types in the brain aside from the more widely known neurons. These different cell types are in constant communication with each other and neurons, and neural function depends on these interactions. One of the largest families of these cells in humans are called astrocytes, named after their complex "star-like" morphology. A number of important housekeeping functions are carried out by astrocytes, including clearance of the excitatory neurotransmitter glutamate from the external environment surrounding neurons. This clearance is important as glutamate outside of neurons is toxic and can lead to neuronal death. Furthermore, astrocytes form close associations with the information transfer points, called synapses, between neurons, and are able to detect and respond to neuronal information passing through these points. This has led to the suggestion that astrocytes may play a direct role in neuronal communication on top of their housekeeping duties. However, the extent of the communication between astrocytes and neurons, and the functional consequences of this, are only just beginning to be explored.

My thesis addresses this gap in knowledge, revealing how neurons control astrocyte properties, and how astrocytes in turn control neuronal properties. First, I show that neurons send signals to astrocytes through a contact-mediated pathway that gives astrocytes the ability to clear glutamate. I show that this neuronal signalling is required to maintain astrocytic glutamate clearance, which has consequences for neurodegenerative disease, where contact between neurons and astrocytes is lost. I then reveal a list of neuronal genes whose expression is controlled by astrocytes. I find that a group of genes for the functionally important potassium channels are reduced in neurons by a factor secreted by astrocytes. I show that this reduction in potassium channels alters neuronal membrane properties, making neurons more excitable and active. These potassium channels and neuronal excitability are regulated in response to abnormal stimulation in order to maintain correct neuronal activity, in a process known as homeostasis. Importantly, I show that without astrocytes, neurons are not capable of this homeostatic response that is essential in order to mitigate pathological activity levels and maintain brain function.

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Abbreviations

ACM	Astrocyte conditioned media
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid hydrobromide
apoE	Apolipoprotein E
AraC	Cytosine arabinoside
CAM	Cell adhesion molecule
cAMP	Cyclic AMP
CC	Co-culture
Chrdl1	Chordin-like 1
CNS	Central nervous system
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DIV	Day *in vitro*
EAAT	Excitatory amino acid transporter
EF-GFP	Enhanced fluorescence GFP
EM	Electron microscopy
EPSC	Excitatory post-synaptic current
FI	Frequency-current
GABA	γ-Aminobutyric acid
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GS	Glutamine synthetase
GP	Glypican
IV	Current-voltage
K_{IR}	K^+ inward rectifier channel
L-Asp	L-Aspartate
LTD	Long-term depression
LTP	Long-term potentiation
mAChR	Muscarinic receptor
MAPK	Mitogen-activated protein kinases
MC	Mono-culture
mEPSC	Miniature EPSC
mGluR	Metabotropic glutamate receptor
mRNA	Messenger RNA
NCM	Neuron conditioned media

NICD	Notch intracellular domain
NL	Neuroligin
NMDA	N-Methyl-D-aspartic acid
PAG	Phosphate-activated glutaminase
PCR	Polymerase chain reaction
PTX	Picrotoxin
RGC	Retinal ganglion cell
R_M	Membrane resistance
RMP	Resting membrane potential
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription PCR
TFB-TBOA	(3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid
TLE	Temporal lobe epilepsy
t-LTD	Spike-timing dependent LTD
TQ	Tertiapin Q
TSP	Thrombospondin
TTX	Tetrodotoxin

Chapter 1

Introduction

Chapter 1 - Introduction

1.1 An introduction to the interplay of neurons and astrocytes

For many years the study of the brain focused almost solely on the communication between one family of cells in the brain, the neurons. Despite being aware of other central nervous system (CNS) cell types for over a century, the pronounced electrical responses of the neurons made them the focus of study, whilst the other cell types were side-lined, with the belief they provided little more than structural support (Kettenmann and Verkhratsky, 2008, Weigert, 1895, Ramon-y-Cajal et al., 1996). This historical bias is reflected today with non-neuronal cells still collectively termed glia, meaning glue, whereas neurons have become the emblem for our field: *neuro*science.

Recently this bias has begun to be addressed, with research into the different glial cells, including the oligodendrocytes, microglia, astrocytes and $NG2^+$ cells, rapidly gaining momentum. It is increasingly clear that all of the CNS cell types play an important role in nervous system function, with the interactions between the different glia types and neurons being fundamental to successful neuronal development, health, information processing and storage.

An abundant and formerly overlooked cell family in the nervous system are the astrocytes. In the human cortex astrocytes represent an estimated 10-12% of all cells present, although most attempts to count cell numbers have not thought it necessary to differentiate between glial cell types, so it is still uncertain the exact proportion of astrocytes in different regions of the brain (von Bartheld et al., 2016, Pelvig et al., 2008). Astrocytes are derived from the same neural precursor cell population, radial glia, as neurons. Developmentally, in the early embryonic stages radial glia precursor cells are derived from basal neuroepithelium cells, and generate neurons from around embryonic day 10 (E10) to E18 (in rodents) (Haubensak et al., 2004, Miller and Gauthier, 2007). Around this point there is a "gliogenic switch" triggered by various signalling cascades, including neuron to radial glia Notch signalling, whereupon radial glia begin producing astrocytes (and oligodendrocytes) instead of neurons (Miller and Gauthier, 2007, Rowitch and Kriegstein, 2010). During maturation astrocytes develop bushy protrusions enveloping large volumes, which extend and connect with their neighbouring astrocytes' protrusions through gap junctions to create a "tiled" astrocyte

network throughout the brain, as well as extending their processes out to neuronal synapses (Bushong et al., 2004, Ventura and Harris, 1999).

1.1.1 Passive astrocyte-neuron interactions

Given astrocytes' abundance, complex structures and close associations with synapses, you would expect these cells to play an important role in the brain. Indeed, from the 1960s it was observed and subsequently confirmed that astrocytes play an important role in K^+ buffering, and from the 1990s that they are the primary collectors of neuronally released glutamate (Orkand et al., 1966, Hertz, 1965, Walz, 2000, Rothstein et al., 1996, Danbolt, 2001, Brown, 2017). As either elevated extracellular K^+ or glutamate concentrations can lead to neuronal hyperexcitability and death, this was the first work demonstrating an important role of astrocytes for neuronal health and function. Additionally, astrocytes are now established players in antioxidant defence, producing glutathione for the reduction of peroxides, and there is strong debate whether astrocytes provide metabolic substrates, such as lactate, to sustain high neuronal energy requirements (Calì et al., 2019, Dienel, 2018, Dringen et al., 2005, Hirrlinger et al., 2002, Baxter and Hardingham, 2016). From this work astrocytes are now well established as providers of homeostatic support for neurons.

1.1.1.1 Control of extracellular environment

Today, the most well-established function of astrocytes remains their buffering of K^+ and their clearance of extracellular glutamate, which maintains the concentrations of these stimulating factors throughout the extracellular environment. During an action potential, K^+ is released from neurons, increasing the extracellular K^+ concentration (Frankenhaeuser and Hodgkin, 1956). Due to the expression of numerous K^+ channels in cellular membranes, increases in extracellular K^+ can further depolarise membrane potentials, which can lead to aberrant neuronal activity. As such, extracellular K^+ concentration is tightly regulated, typically increasing by less than 1 mM following normal activity, with the astrocyte network being a central component for this maintenance (Syková, 1983, Larsen et al., 2016). Although the exact mechanism is still debated, astrocytes respond to increases in extracellular K^+ by first collecting it through both astrocytic Na^+, K^+-ATPase transporters and their extensively expressed K^+ inward rectifier channel, $K_{IR}4.1$ (Larsen et al., 2014). This sequestered K^+ spreads throughout the astrocyte network, at least partially through astrocytic gap junctions, and is then released back out again into areas of low K^+ concentration through $K_{IR}4.1$ channels (Wallraff

et al., 2006, Hertz and Chen, 2016). As well as their regulation of K^+ concentration, astrocytes are also responsible for maintaining the exceptionally low extracellular levels of glutamate in the brain required to prevent neuronal excitotoxicity, through their high affinity glutamate transporters (Danbolt, 2001). I expand on this subject matter in detail throughout *Chapter 1.3-1.5* of the introduction, but it appears that the expression of these functionally crucial transporters in astrocytes is regulated by neurons (Schlag et al., 1998).

1.1.1.2 Antioxidant provision

One of the first observations of astrocytic influence on neurons was the enhanced survival of neurons grown in the presence of astrocytes. As well as the astrocytes' role in preventing excitotoxicity by their buffering of K^+ and removal of glutamate via their glutamate transporters, they are further involved in neuroprotection via the provision of antioxidants. The brain, and in particular the neurons within, is a highly metabolically active organ, using 20% of the total body energy and O_2 consumption despite making up only 2% of total body mass (Dienel, 2018). This oxygen consumption results in the generation of peroxides and other derived reactive oxygen species (ROS), which can cause DNA damage, initiate faulty cell signalling and induce apoptotic pathways if not sufficiently neutralised (Barnham et al., 2004, Dringen et al., 2005). There are several antioxidant pathways responsible for clearing up these reactive species, including glutathione, catalase, superoxide dismutase and thioredoxin, and it has become apparent that astrocytes are heavily involved in this process (Baxter and Hardingham, 2016).

One of the major pathways for peroxide neutralisation in the brain is the glutathione system. In this pathway, H_2O_2 is reduced with glutathione by glutathione peroxidase into water and glutathione disulphide, which can then be converted back to glutathione via glutathione reductase (Dringen et al., 2005). Using neuron or astrocyte cultures it was observed that this pathway is endogenously active in astrocytes rather than neurons (Dringen et al., 1999). Furthermore, the glutathione required for this detoxification pathway is the product of nuclear factor erythroid-2-related factor 2 (Nrf2) activation of the antioxidant response element (ARE), with genes for this glutathione pathway being specifically upregulated in astrocytes rather than neurons following oxidative stress (Bell et al., 2011, Vargas and Johnson, 2009).

As neurons are the major consumers of oxygen, and hence producers of ROS, glutathione is greatly needed in neurons themselves to prevent peroxide mediated toxicity. Astrocytes therefore would need to send this glutathione across to neurons. Indeed, evidence suggests that astrocytes export glutathione into the extracellular environment, where it is converted into cysteine, and this cysteine can then be taken up by neuronal xCT transporters where it is used (along with astrocyte-derived glutamine) as a precursor for neuronal glutathione production (Hirrlinger et al., 2002, Shih et al., 2003, Vargas and Johnson, 2009). In this way astrocytes not only provide neuroprotection via clearance of K^+ and glutamate, they also respond to oxidative stress, producing antioxidants that neurons can then use for their protection.

1.1.1.3 Metabolic support of activity

Neuronal activity has one of the largest energy requirements in the body, most of which goes towards supporting synaptic transmission (Harris et al., 2012, Dienel, 2018). Unlike other tissues, the brain is almost solely dependent on glucose to support this activity under normal conditions, with the human brain requiring approximately 90 g of glucose per day (Chugani et al., 1987, Madsen et al., 1995). Over two decades ago it was proposed that in response to neuronal activity astrocytes increase their uptake of glucose from blood vessels and convert it into lactate, which they then shuttle to neurons to use as fuel, in what was termed the astrocyte-neuron lactate shuttle (Pellerin et al., 1998). This proposal has been widely debated and the extent to which neurons use astrocyte provided lactate as a metabolic substrate is under question (Bélanger et al., 2011, Boumezbeur et al., 2010, Dienel, 2017). It is likely that neurons preferentially metabolise glucose under basal conditions, but under higher metabolic demands astrocytes are able to increase lactate production and release as an alternative energy source for neurons.

A second key way in which astrocytes support the high energetic demands of neurons is by providing them with amino acid precursors such as glutamine for the production of both glutamate and γ-aminobutyric acid (GABA), a key feature of the glutamate-glutamine cycle which will be discussed in greater detail later in this chapter. Surprisingly, neurons express little of the enzyme pyruvate carboxylase, giving them a limited capacity for the *de novo* synthesis of metabolic intermediates that are required for glutamate and GABA synthesis, whereas astrocytes express this enzyme in abundance rendering them capable of the anaplerotic reactions required (Bak et al., 2006,

Yu et al., 1983). As astrocytes are additionally the major clearers of glutamate, this would result in the run-down of pre-synaptic glutamate stores following excitatory transmission, as neurons neither uptake nor synthesise the neurotransmitter to a great extent. It follows that astrocytes must then supply neurons with the precursor glutamine and/or other metabolic intermediates generated through astrocytic anaplerosis in order for neuronal neurotransmitter stores to be replenished, allowing transmission to continue.

The requirement of astrocytic glutamine for maintaining excitatory activity was nicely demonstrated several years ago by Reimer and colleagues (Tani et al., 2014). In hippocampal slices the authors showed that under a low stimulus paradigm (0.2 Hz activity bursts), preventing astrocytic conversion of glutamate to glutamine by inhibition of the enzyme glutamine synthetase (GS) had little effect on neuronal activity. In the hippocampus 20-30 Hz of activity can be observed in mice *in vivo* during exploration, so the authors then increased the stimulus to 2 Hz, still a submaximal stimulus, and this time found that there was a continual decrease in field amplitude when astrocytic GS was inhibited, that could be prevented by bath applying 500 μM of exogenous glutamine to the aCSF (Tani et al., 2014). When they increased to 20 Hz stimulation, they found a rapid run-down to near full abolishment of field responses when astrocytic glutamine production was inhibited, and exogenous glutamine could only partially prevent the effects. However, this continual high burst activity is un-physiological, so they moved to an intermittent 20 Hz followed by 200 s of low 0.2 Hz stimulation protocol, as well as a physiological protocol based on activity patterns recorded from an *in vivo* freely moving animal. Under these more physiological scenarios, without astrocytic glutamine supply there was an almost complete lack of induced field response after several high frequency events, but in the presence of exogenous glutamine this rundown was completely prevented (Tani et al., 2014). They further demonstrated this was also the case for excitatory pyramidal layer III cortical cells, with intermittent 20 Hz stimulation causing a decreasing field response without astrocytic glutamine, that was recovered by the application of exogenous glutamine (Tani et al., 2014).

As yet, there is no conditional astrocytic glutamine transporter knock down to confirm the necessity of astrocytic glutamine release for maintaining activity, nor any drugs capable of specifically inhibiting the astrocytic glutamine transporter (SNAT3; Todd et al., 2017). A SNAT3 knockout mouse has been reported, but due to the importance of SNAT3 in the kidney this resulted in animal

death by post-natal day 20. However, by this stage the mice had significantly reduced glutamate and GABA, but increased glutamine, concentrations in the brain, as well as a form of ataxia, further supporting the requirement of astrocytic released glutamine for maintaining neuronal glutamate levels required for optimal transmission (Chan et al., 2016).

1.1.2 Active astrocyte-neuron communication

Beyond astrocytes' important but arguably passive roles in homeostasis, astrocytes and neurons are also able to communicate with each other to actively modulate activity and function. The first evidence of neuronal communication to astrocytes came with the advent of Ca^{2+} imaging. Compared to neurons, astrocytes are relatively electrically quiet cells, a contributing factor to the former paucity of research into their function. However, with Ca^{2+} imaging it was shown that astrocytes are highly active cells, showing dynamic and varied Ca^{2+} signalling within their processes, particularly in response to neuronal activity (Porter and McCarthy, 1995, Khakh and McCarthy, 2015). This confirmed that astrocytes both directly sensed and responded to neuronal communication. More recent work has additionally shown Na^{+} to be a second activity-regulated signalling mechanism within astrocytes (Verkhratsky et al., 2019, Kirischuk et al., 2012).

A hot topic of research currently is how astrocytes may communicate back to neurons and alter neuronal function and activity. Evidence suggests that astrocyte to neuron signalling may be important in axon and dendrite growth and formation, as well as synapse formation (Le Roux and Esquenazi, 2002, Baldwin and Eroglu, 2017, Christopherson et al., 2005). Furthermore, the notion of a tripartite synapse has become commonplace, whereby astrocytes act as a third player along with the pre- and post-synapse, releasing neuro-modulatory factors to directly stimulate neuronal synapses (Perea et al., 2009, Parpura et al., 1994, Araque et al., 1998). This direct astrocyte to synapse signalling may be required for various forms of neuronal plasticity, including long-term potentiation (LTP) and long-term depression (LTD) (Henneberger et al., 2010, Min and Nevian, 2012).

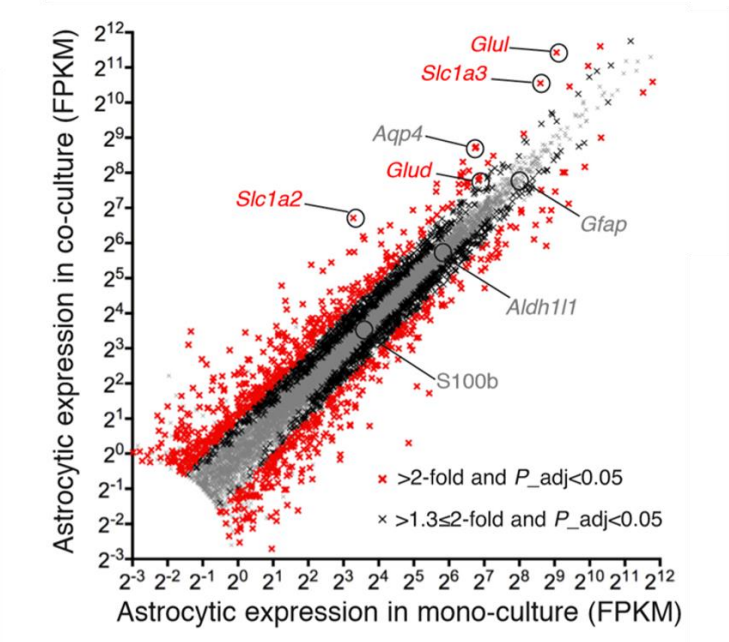
Although there has been a rapid expansion in the knowledge of these more active forms of astrocyte-neuron communication, the field is still in its relative infancy, with many gaps in knowledge still to be addressed. In my thesis I explore both sides of this bidirectional communication between neurons and astrocytes, seeking to answer some of the outstanding questions. First, I investigate

how cortical neurons signal to cortical astrocytes to control astrocytic function, particularly focussing on how neurons control the functionally important astrocytic glutamate clearance pathway. Secondly, I investigate how cortical astrocytes alter cortical neuronal gene expression, and what the functional outcomes of this control are. For the remainder of this introductory chapter I will elaborate on the background literature leading to the work in this thesis.

1.2 Neuron-to-astrocyte communication

It is clear that neurons signal to astrocytes, causing changes in astrocytic form and function. The earliest suggestions that neurons actively communicate to astrocytes came from the observation that astrocytes express a number of receptors for neurotransmitters, and that application of the neurotransmitters glutamate or GABA induced astrocytic depolarisations (Kettenmann and Schachner, 1985, Bowman and Kimelberg, 1984, Murphy and Pearce, 1987). One of the most striking examples of neuron-to-astrocyte signalling is the dramatic morphological changes *in vitro* astrocytes undergo when cultured with neurons, being transformed from flat "pancake" shaped cells into more complex "star" like structures, reminiscent of their *in vivo* forms (Hatten, 1985, Hasel et al., 2017). But despite these observations over three decades ago, the signalling cascades induced in astrocytes by neuronal input, and the functional outcomes, are still largely unknown.

Up until recently, only a few astrocytic proteins were known to be regulated by neurons. In 1997 Swanson and colleagues showed that the two astrocytic glutamate transporters, excitatory amino acid transporter 1 (EAAT1; *Slc1a3*) and EAAT2 (*Slc1a2*), were upregulated by neurons. In cortical astrocytes cultured alone they detected EAAT1 in most astrocytes but little to no EAAT2, but in the presence of neurons they saw a significant increase in EAAT1 along with a strong induction of EAAT2 expression (Swanson et al., 1997). A few years on the connexin 43 (*Gja1*) protein, which forms astrocytic gap functions, was also shown to be upregulated by neurons (Rouach et al., 2000). Additionally, in 1988 glutamine synthetase metabolic activity appeared to be increased in forebrain astrocytes grown in the presence of forebrain neurons, suggesting that the interaction between neurons and astrocytes may also regulate astrocytic glutamine synthetase (Hayashi et al., 1988). However, even for just these few known targets, the neuronal signals behind their regulation in astrocytes are still largely unknown.



	$Astrocyte_{Ms}$ monoculture	$Astrocyte_{Ms}$-$Neuron_{rat}$ coculture
Average no. of reads mapped to mouse genome per sample (n = 3)	38,056,262	13,380,921
% of mapped reads attributed to the mouse genome	99.9	12.4
Genes expressed >0.5 FPKM	13,122	13,163
Differentially expressed genes (≥2-fold difference)	763 (5.8 %)	
Differentially expressed genes (≥1.3-fold difference)	2,116 (16.1 %)	

Figure 1.1: Cortical rat neuronal control of cortical mouse astrocytic transcriptome

Neurons regulate the expression of astrocytic genes, with approximately 16 % of cortical (mouse) astrocytic genes being significantly up or downregulated by ≥1.3-fold by rat neurons. Prominently amongst the upregulated mouse genes are those for glutamate uptake, *Slc1a3* (EAAT1) and *Slc1a2* (EAAT2), and glutamate metabolism, *Glul* (glutamine synthetase) and *Glud* (glutamate dehydrogenase). Commonly used astrocytic markers, such as *S100β*, *Aldh1l1* and glial fibrillary acidic protein (*Gfap*) were unaffected by co-culture with neurons, although the astrocytic water channel (*Aqp4*) was significantly upregulated. Figure adapted from Hasel et al., 2017.

To address this gap in knowledge our laboratory recently undertook RNA-sequencing (RNA-seq) of cortical astrocytes grown alone or in the presence of cortical neurons (Hasel et al., 2017). We uncovered hundreds of previously unknown astrocytic genes that are regulated by neurons, for example the astrocytic water channel, aquaporin 4 (*Aqp4*; see *Figure 1.1*). However, one of the strongest effects of neurons on astrocytes that we saw was the induction of the already reported astrocytic glutamate transporters, *Slc1a2* and *Slc1a3*, as well as genes for proteins involved in

glutamate metabolism, *Glul* (glutamine synthetase) and *Glud* (glutamate dehydrogenase). Given the functional importance of glutamate clearance and recycling, I chose to focus on exploring how neurons control astrocytic glutamate transporter function, and the signalling pathway involved.

1.3 Astrocytic glutamate clearance and recycling

Glutamate is the predominant neurotransmitter in the brain, activating post-synaptic ionotropic N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors. Co-activation of AMPA and NMDA receptors allows the influx of Na^+ ions, depolarising the cell's membrane potential, with sufficient depolarisation triggering an action potential. As well as being located on the post-synaptic density, NMDA receptors can be found in extrasynaptic locations. This is significant as NMDA receptors allow Ca^{2+} to pass through their channel pore as well as Na^+, with this Ca^{2+} causing the induction of different signalling cascades depending on both the NMDA receptor location and subtype (Hardingham et al., 2002, Hardingham and Bading, 2010, Martel et al., 2012). As well as activating ionotropic receptors, glutamate also activates the metabotropic glutamate receptor family (mGluRs), which are G-protein coupled receptors (GPCR), whose stimulation initiates various signalling cascades (Gerber et al., 2007).

Once released it is important that glutamate is cleared away, as too much glutamate in the extracellular space becomes neurotoxic. Firstly, if it isn't cleared away it will continue to stimulate the post-synaptic receptors after the initial signal has been sent, impairing the detection of the next signal that arrives and potentially leading to cell swelling due to ion influx (Danbolt, 2001). Secondly, if this glutamate escapes from the synaptic zone it was released into it could activate unintended synapses, triggering activity where it should not. But most significantly, if glutamate escapes the synaptic region it can activate extrasynaptic NMDA receptors: too much Ca^{2+} influx via these extrasynaptic NMDA receptors induces signalling cascades that initiate cell death programs (Hardingham and Bading, 2010).

Astrocytes have long been known to be important in promoting the survival of neurons and for counteracting the toxic effects of glutamate (Lindsay, 1979, Banker, 1980, Rosenberg et al., 1992, Rosenberg and Aizenman, 1989). This protection is largely due to the fact they are predominantly

responsible for clearing away glutamate via transporters located on astrocytic membranes, preventing excitotoxicity. Once inside the astrocytes, the glutamate is then either converted into α-ketoglutarate by glutamate dehydrogenase (GDH) or transaminases and shunted into the astrocytic TCA cycle, or else converted into glutamine by the enzyme glutamine synthetase (GS) (Hertz and Rodrigues, 2014, Sonnewald and Schousboe, 2016). Glutamine is not toxic to neurons, and is extruded by the SNAT3 glutamine transporter into the extrasynaptic space, which can then be taken up by neurons and converted back into glutamate via the neuronally expressed phosphate-activated glutaminase (PAG), thus replenishing pre-synaptic glutamate stores (Billups et al., 2013, Tani et al., 2014, Todd et al., 2017, Uwechue et al., 2012). This cycle is referred to as the glutamine-glutamate cycle (*Figure 1.2*).

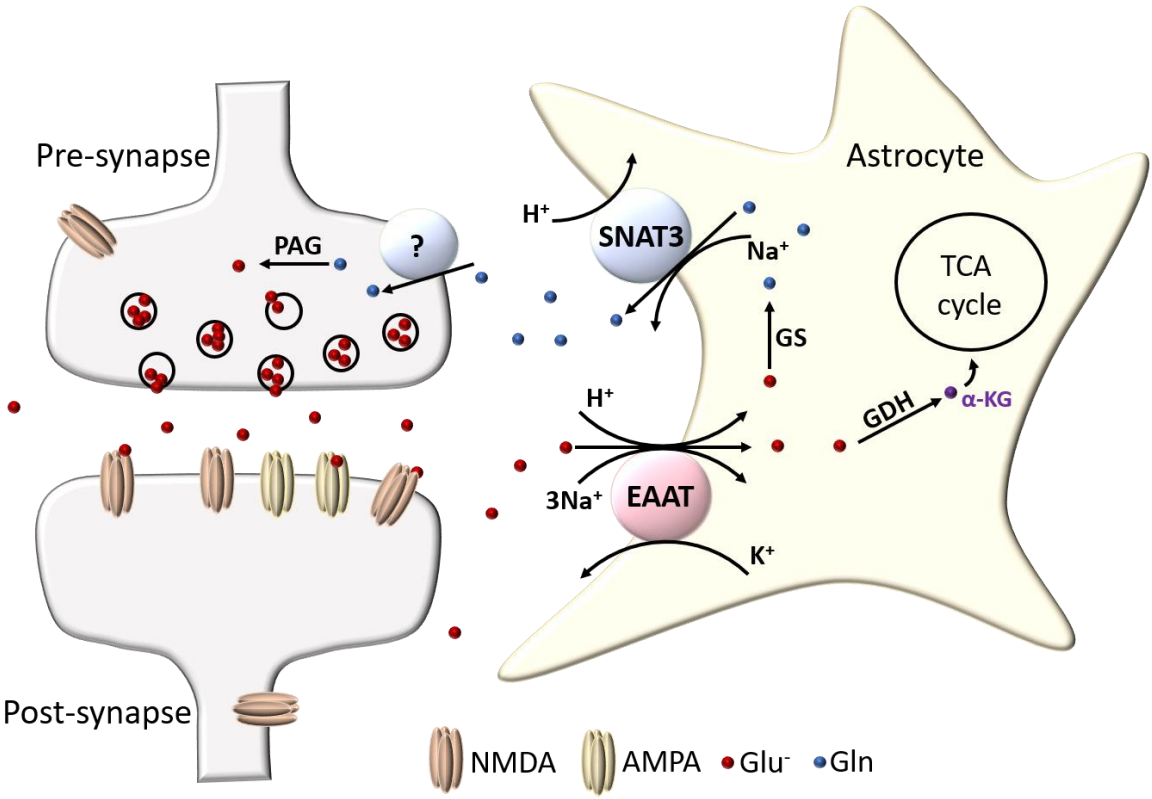


Figure 1.2: The glutamine-glutamate cycle

Glutamate (Glu^-) released after excitatory transmission is collected by astrocytic EAAT transporters 1 & 2. The glutamate is then either converted into α-ketoglutarate (α-KG) via glutamate dehydrogenase (GDH) or transaminase reaction and enters the TCA cycle, or else is converted into glutamine (Gln) by glutamine synthetase (GS). Astrocytes excrete Gln back into the extracellular environment via the Na^+ driven SNAT3 transporter, which is then taken up by an as yet unconfirmed neuronal Gln transporter. Neurons then convert Gln back to Glu^- via a phosphate-activated glutaminase (PAG) reaction to replenish their vesicular Glu^- stores. Adapted from Todd et al., 2017.

Astrocytic glutamate uptake and recycling is a vital part of CNS function, with impairments in this system associated with many nervous system diseases. Key within this machinery are the two glutamate transporters, excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2), that are responsible for the astrocytic uptake of glutamate.

1.4 Glutamate transport within the brain

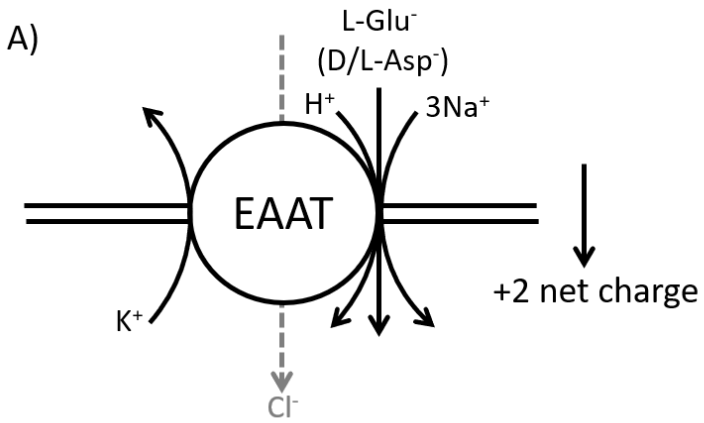
Glutamate is found at high concentrations in the brain, at a concentration of approximately 10 – 14 mM/L depending on region (Hädel et al., 2013, Schubert et al., 2004). However, most of this glutamate is kept within intracellular compartments, with only 3–4 μM/L found in the extracellular fluid (Lehmann et al., 1983, Hamberger and Nyström, 1984). Accordingly, there are numerous transporter proteins in the brain that are capable of facilitating glutamate transport to ensure that the right concentration of glutamate is maintained in the right compartments. These transporters first fall into two broad categories: those which are found in intracellular compartments, such as the three vesicular glutamate transporters (vGLUT1-3) which package glutamate into synaptic vesicles, and those located on the plasma membrane of cells that can transport glutamate into (or out of) the cell (see Danbolt, 2001, Erecińska and Silver, 1990, Helms et al., 2017, for reviews). The glutamate transporters that are found in the plasma membranes of brain cells consist of five sodium-dependent co-transporters, and one sodium-independent exchanger (Fotiadis et al., 2013, Grewer et al., 2014, Kanai et al., 2013). The sodium-independent exchanger, xCT, is found almost exclusively on astrocytes, but preferentially transports cysteine into the cell in exchange for extruding a glutamate molecule out of the astrocyte (Bridges et al., 2012, Ottestad-Hansen et al., 2018). Due to the need to transport glutamate into cells against its electrochemical gradient, it is therefore the sodium-dependent class of transporters that are responsible for quickly sequestering extracellular glutamate back into cells. There are five known members of this family of transporters, excitatory amino acid transporters 1 – 5.

1.4.1 The excitatory amino acid transporters

In the early 1970s a high affinity sodium-dependent uptake system for the negatively charged amino acids L-glutamate and L-aspartate was first described in synaptosomal preparations, which was hypothesised to be responsible for the accumulation of the putative excitatory neurotransmitter

glutamate into cells (Logan and Snyder, 1971, Balcar and Johnston, 1972). A few years later Balcar and colleagues went on to show that this glutamate uptake system was also present in glial cells, but not until 1992 were EAATs first purified, with four independent groups cloning three distinct EAAT family members: Glt-1 (EAAT2), GLAST (EAAT1), and EAAC (EAAT3) (Kanai and Hediger, 1992, Pines et al., 1992, Storck et al., 1992, Balcar et al., 1977, Tanaka, 1993). The final two members were cloned in 1995 (EAAT4) and 1997 (EAAT5) (Fairman et al., 1995, Arriza et al., 1997).

A)



B)

	Gene	Cl^- conductance	Location
EAAT1 (GLAST)	Slc1a3	Moderate	Astrocytes (incl. Bergmann & Müller glia); Predominant EAAT subtype in cerebellum (1.8 mg/g) and retina
EAAT2 (Glt-1)	Slc1a2	Low	Astrocytes (and some sparse neurons); Predominant EAAT subtype in hippocampus (1.3 mg/g) and cortex (0.8 mg/g)
EAAT3 (EAAC)	Slc1a1	Moderate	Neurons (typically on spines); Highest concentration in hippocampus (0.013 mg/g)
EAAT4	Slc1a6	High	Cerebellar Purkinje cells (0.2 mg/g of protein in cerebellar molecular layer)
EAAT5	Slc1a7	High	Retina (rod photo receptors, bipolar cells)

Figure 1.3: Excitatory amino acid transporter family

A) Stoichiometry of EAAT family transporters. EAATs can function as an anion channel, with the associated Cl^- conductance uncoupled to the transport of glutamate. B) Overview of the five EAAT family members. References: (Lehre et al., 1995, Dehnes et al., 1998, Lehre et al., 1997, Lehre and Danbolt, 1998, Holmseth et al., 2012, Massie et al., 2008, Pow and Barnett, 2000, Wersinger et al., 2006).

All members of the EAAT family transport L-glutamate into cells under normal conditions using the electrochemical gradients of Na^+ and K^+ (Danbolt, 2001, Grewer et al., 2014). The different subtypes are found throughout the body, and within the brain they are found on different cell types and in different brain regions. Although they all transport glutamate into cells, each subtype possesses a different degree of chloride permeability, and it appears the function of each of these subtypes may vary. A summary of the five EAAT transporters is given in *Figure 1.3*.

1.4.1.1 Location

The two subtypes EAAT1 (analogous to GLAST) and EAAT2 (analogous to Glt-1) are referred to as the astrocytic glutamate transporters as they are the only EAAT subtypes expressed on astrocytes, where they are predominantly found on fine astrocytic processes opposed to glutamatergic synapses (Šerý et al., 2015, Zhou and Danbolt, 2013, Chaudhry et al., 1995). EAAT1 is found in astroglia (including the Bergmann and Müller glia) throughout the brain, on which they are exclusively expressed, and are the primary collectors of glutamate in both the cerebellum and retina (via Bergmann and Müller glia, respectively) (Lehre and Danbolt, 1998, Lehre et al., 1995, Schmitt et al., 1997, Rothstein et al., 1994, Derouiche and Rauen, 1995, Lehre et al., 1997, Pow and Barnett, 1999). EAAT2, on the other hand, is the main glutamate transporter in other brain regions, but most prominently in the hippocampus and cortex (Lehre et al., 1995, Zhou and Danbolt, 2013). The location of EAAT2 is less astrocyte-exclusive, with some evidence suggesting it is also found to a small degree in neurons, particularly in the hippocampus and retina (see Zhou and Danbolt, 2013 for discussion). Combined, EAAT1 and EAAT2 make up a significant proportion of the total protein in the brain, representing ~2.1% of protein in the molecular layer of the cerebellum, 1.6% in the hippocampal *stratum radiatum*, and 1% of protein in forebrain tissue, making them the most abundant EAATs found in the CNS, and clearly of functional importance (Lehre and Danbolt, 1998).

The EAAT3 subtype is exclusively neuronal, and is found on neurons throughout the brain (Holmseth et al., 2012, Shashidharan et al., 1997). It typically localises on dendritic spines, and not axon terminals, with its highest expression seen in the hippocampus, at a concentration of 0.013 mg/g (Holmseth et al., 2012). This is 100 times less than the concentration of EAAT2 in the

same region, so even here is unlikely to significantly contribute to glutamate clearance. EAAT4 is another neuronally expressed glutamate transporter, however its expression profile is more restricted than that of EAAT3, being found primarily on Purkinje cells of the cerebellum, with some sparse expression in certain subregions of the forebrain and midbrain (Dehnes et al., 1998, Massie et al., 2008). It represents about 0.2% of protein in the molecular layer of the cerebellum, which is approximately 10 times less than the predominant astroglial subtype in this region, EAAT1 (which represents 1.8% of total cerebellar protein), and about par with the EAAT2 subtype (sitting at 0.3%) (Dehnes et al., 1998, Lehre and Danbolt, 1998). The final member of the family, EAAT5, has only been found in the eye, where it is located on synaptic terminals of retinal rod bipolar cells as well as rod and cone photoreceptors (Arriza et al., 1997, Pow and Barnett, 2000). Again, the astrocytic EAAT1 subtype is expressed more strongly in the retina than EAAT5, and there is evidence that EAAT5 may physiologically act as a chloride channel rather than a glutamate transporter in these retinal neurons (Eliasof et al., 1998, Wersinger et al., 2006).

1.4.1.2 Structure and function

All EAATs support the transport of L-glutamate as well as D and L-Aspartate, displaying a relatively high affinity for L-glutamate, with reported K_M for glutamate ranging from 10-100 μM (Arriza et al., 1997, Arriza et al., 1994, Gegelashvili and Schousboe, 1998). Given that the glutamate concentration within cells is many-fold higher than that in the extracellular space, combined with the fact that glutamate is an anion carrying -1 charge, the stoichiometry of the EAATs must be such that the transport cycle overcomes the electrochemical gradient of glutamate. The stoichiometry was debated for some time, with different groups arguing for whether the transporters couple two or three Na^+ ions to the transport of one glutamate (Zerangue and Kavanaugh, 1996, Kanai et al., 1995). It has since been established that the EAATs combine the transport of 1 glutamate molecule with the co-transport of 3 Na^+ and 1 H^+, whilst counter-transporting 1 K^+, as shown in *Figure 1.3* (Levy et al., 1998). This has been supported by more recent studies using the homologous archaeal glutamate transporters Glt_{Ph} and Glt_{Tk} whose structures were crystallised in 2004 and 2013 (Yernool et al., 2004, Jensen et al., 2013, Arkhipova et al., 2019, Kortzak et al., 2019). As a result, the transport cycle is charged, with a net +2 charge per molecule of glutamate transported, facilitating the inward movement of the otherwise negatively charged glutamate, whilst using the Na^+ and K^+ concentration gradients to further drive transport into the cell. Importantly, this stoichiometry is

estimated to allow the internal glutamate concentration to be in the order of 10^6 times greater than the external concentration under physiological conditions, ensuring the transporters work to take up rather than extrude glutamate under normal conditions (Danbolt, 2001).

Although this transporter stoichiometry is believed to be common to all members of the EAAT family, meaning all EAATs could help facilitate glutamate clearance, there is a difference between the EAATs. As well as functioning as a transporter, EAATs can also act as ligand-gated ion channels, with glutamate activation leading to an uncoupled conductance of Cl^- through the channel (Wadiche et al., 1995, Wadiche and Kavanaugh, 1998, Fairman et al., 1995, Machtens et al., 2015). However, the level of anion conductance varies largely between the different subtypes (Fahlke et al., 2016). EAAT4 and EAAT5 display the largest ion conductance, with their Cl^- conductance being greater than that of their glutamate uptake, EAAT1 and EAAT3 have intermediate ion conductance, while EAAT2 displays very little conductance at all (Arriza et al., 1997, Fairman et al., 1995, Kanai et al., 2013, Wadiche et al., 1995). Recent work has suggested that EAAT5 uses this anionic conductance to act as an "inhibitory" glutamate receptor in retinal cells, hyperpolarising these cells' membrane potentials following glutamate activation (Schneider et al., 2014, Tse et al., 2014, Wersinger et al., 2006). It is likely that both the EAAT4 and EAAT5 subtypes do not physiologically function as glutamate uptake systems, but instead act as Cl^- channels.

The first support for astrocytic glutamate transporters EAAT1 and EAAT2 being responsible for glutamate clearance rather than neuronal subtypes came from studies using autoradiographic localization, which found the bulk of cleared glutamate was seen in glial cells (McLennan, 1976, Wilkin et al., 1982). Electrophysiological recordings later went on to show that this astrocytic glutamate clearance was mediated by EAAT1 and EAAT2 (Bergles and Jahr, 1998, Bergles and Jahr, 1997). Final evidence that it is the astrocytic EAAT1 and EAAT2 subtypes, and not the neuronal EAAT3, that are responsible for glutamate clearance comes from knockout studies.

An EAAT2 knockout animal was generated in 1997, which developed lethal seizures resulting in an 80% death rate by 13 weeks of age, compared to 100% survival in controls (Tanaka et al., 1997). It was found that there was a slower clearance of synaptically released glutamate in knockout animals, with neuronal degeneration appearing specifically in the hippocampal CA1 region (Tanaka et al., 1997). In 1998 the group went on to generate an EAAT1 knockout animal (Watase et al., 1998).

Unlike the EAAT2 knockout, removal of EAAT1 did not appear to be lethal, and brain development appeared normal. The group focused on the cerebellum, given that is EAAT1's prominent region of expression, and found that in EAAT1 knockouts glutamate uptake in this region was nearly half that of wild-types. Although finding no difference in basic motor tasks, they found a significant impairment in the knockouts' ability to complete a more challenging rotor-rod experiment. Further, they found that mutant EAAT1 animals, but not wild-types, were susceptible to cerebellar edema following cold injury (Watase et al., 1998). Inevitably, in 2006 the group reported on a double EAAT1/EAAT2 knockout animal. Unlike the single mutants, the double knockout of EAAT1 and EAAT2 was embryonic lethal, with mice dying by E17-18, and brain-wide abnormalities in structure observed (Matsugami et al., 2006). These studies highlight the vital importance of this system in the CNS.

In 1997 a second group generated a mouse knockout for the neuronal glutamate uptake transporter, EAAT3 (Peghini et al., 1997). Contrary to the neurological deficits seen in EAAT1 or EAAT2 knockout animals, removal of the neuronal glutamate uptake transporter, EAAT3, had no negative effect on brain formation or function over a period of >12 months. There was no impairment in motor skills, nor in memory, nor in susceptibility to induced seizures (Peghini et al., 1997). The group did observe that the mutant mice developed dicarboxylic aminoaciduria, although this is attributed to the fact that EAAT3 plays an important role in glutamate transport in the kidneys. A limitation of all these studies, particularly for the EAAT2 knockout, is in the fact they were global knockout models, and not astrocyte specific. As EAAT2 is reportedly expressed on neurons, this does not rule out neuronal EAAT2 glutamate uptake as being an important source of glutamate clearance to prevent excitotoxicity. Addressing this limitation, Rosenberg and colleagues produced conditional neuronal and conditional astrocytic EAAT2 knockout lines (Petr et al., 2015). Whilst neuronal knockouts showed no difference in growth and lifespan, astrocytic EAAT2 mutants had lower weight gain and significantly higher mortality rates compared to controls (Petr et al., 2015). In proteoliposome preparations from forebrains they found glutamate uptake in astrocytic mutants was 25% of that in controls, whereas there was no difference in glutamate uptake in these preparations between neuronal knockouts and controls. EEG recordings further showed astrocytic EAAT2 knockouts to have significantly more seizure events than controls, with no difference between the conditional neuronal knockouts and controls (Petr et al., 2015).

Altogether, the evidence shows that it is EAAT1 and EAAT2 expressed on astrocytes that are primarily responsible for clearing extracellular glutamate to prevent excitotoxicity. The stoichiometry of the EAAT transporters provides one explanation for why it is the role of astrocytes and not neurons: uptake can result in significant depolarisation (up to 2+ per molecule). If neurons were required to take up the bulk of released glutamate, this process in itself would cause significant neuronal depolarisation, potentially leading to a hyper-excitable feedback loop. Additionally, uptake would result in a significant increase in internal Na^+ concentration, which is counterproductive to the neuron's need to remove internal Na^+ following an action potential and would represent a major metabolic strain on the already metabolically greedy neurons.

1.4.2 Impairments in astrocytic glutamate clearance are features of disease

Unsurprisingly, impairments in the maintenance of extracellular brain glutamate concentration is observed in numerous diseases. A number of accounts have linked astrocytic glutamate transporter dysfunction both to epilepsy and the neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), multiple sclerosis, Alzheimer's disease and Parkinson's disease. In addition, there are also suggestions that dysfunction in these transporters might be features of depression and other emotional disorders.

1.4.2.1 Astrocyte glutamate transporters and epilepsy

The first demonstration that increased glutamate concentrations are a feature of epilepsy came from the results of an *in vivo* microdialysis investigation into the concentrations of GABA and glutamate in the hippocampi of epilepsy patients from 1989 to 1992 (During and Spencer, 1993). The investigators found that an increase in glutamate concentration appeared in the epileptogenic hippocampus approximately 1.5 minutes prior to seizure onset, but not in the contralateral hippocampus. At the onset of seizure glutamate levels became further elevated, with concentrations in glutamate also beginning to increase in the opposing hippocampus. Ten minutes post seizure the non-epileptic hippocampus glutamate concentrations had returned to baseline, whereas the epileptic side had persistently elevated glutamate levels >15 minutes post seizure (During and Spencer, 1993). The patients went on to receive surgical resection of the epileptic hippocampus, with microscopy of the removed tissue revealing moderate to severe pyramidal neuron loss throughout the hippocampal tissue along with reactive gliosis (During and Spencer, 1993).

Given the observance of elevated glutamate concentrations in epilepsy, later work investigated the role of the glutamate transporters in epileptic disease. A significant reduction in astrocytic EAAT2 expression was found in patients with temporal lobe epilepsy (TLE) that went on to develop hippocampal sclerosis, but no change was found in EAAT2 expression in patients without neuronal loss (Proper et al., 2002, Mathern et al., 1999). More recently it was reported that there was a decrease in both EAAT1 and EAAT2 in epileptic hippocampi of patients with intractable treatment-resistant TLE (Sarac et al., 2009). It is not known if reduced astrocytic glutamate transporter function initiates some epileptic diseases or exacerbates it, or even if it is simply an outcome of prolonged disease, in humans. However, animal models have shown that removal of functional astrocytic EAAT2, and not neuronal EAAT3, is sufficient to cause severe and lethal epilepsy, demonstrating the possible involvement of astrocytic glutamate transporter dysfunction in the development of epilepsy (Tanaka et al., 1997, Binder and Steinhäuser, 2006).

1.4.2.2 Involvement in neurodegenerative disease

There are numerous neurodegenerative diseases, which are characterised by a progressive loss of neurons. The different diseases are characterised by disease progression and the specific neurons and brain regions that are primarily affected, for example, in motor neuron diseases there is a loss of motor neurons, whereas Parkinson's disease is characterised by a loss of dopaminergic neurons in the *substantia nigra*. As mentioned in *Chapter 1.3*, excessive extracellular glutamate is neurotoxic and can lead to cell death by various mechanisms, and as such is believed to be a factor in the development and progression of the neurodegenerative diseases. Indeed, regardless of the role that insufficient glutamate clearance may play in the acute development and progression of epileptic activity, a frequent long-term outcome in human epileptic patients is neuronal degeneration (presumably from repeated elevations in glutamate concentrations), particularly in patients with reduced hippocampal EAAT2 levels (During and Spencer, 1993, Farrell et al., 2017, Proper et al., 2002). Given that astrocytes are the main glutamate sink, insufficient astrocytic glutamate clearance to meet requirements is therefore one potential pathway to increased glutamate concentrations and resulting neuronal death. Therefore, much research has gone into investigating the role of astrocytes and their glutamate clearance in different neurodegenerative diseases, with some unequivocal links to disease emerging.

One prominent example is in ALS, a motor neuron disease characterised by progressive loss of motor neurons in the motor cortex, somatosensory cortex and spinal cord, with around 90% of cases occurring sporadically and 10% with familial linkage. From 1992 it was discovered that impaired glutamate uptake in motor regions was a feature of tissue samples from patients with sporadic ALS, and in 1995 that there was a pronounced reduction specifically in EAAT2 protein levels in these tissue samples (Rothstein et al., 1992, Rothstein et al., 1995). Additionally, one patient with sporadic ALS was found to have a mutation in the *SLC1A2* gene that resulted in an EAAT2 protein with reduced glutamate transporter activity, suggesting EAAT2 dysfunction may cause some cases of disease (Trotti et al., 2001). Familial forms of ALS on the other hand were found to be associated with mutations in the superoxide dismutase gene (*SOD1*), with SOD1 mutant protein being shown to reduce functional EAAT2 protein levels in animal models by initiating the cleavage of EAAT2 by Caspase-3 (Deng et al., 1993, Rosen et al., 1993, Gibb et al., 2007, Boston-Howes et al., 2006). Specific deletion of *Slc1a2* in the spinal cord of mice was recently shown to be sufficient to lead to motor neuron degeneration by the fifth month of the mice's lives (Sugiyama and Tanaka, 2018). Finally, in work with our collaborators we have found that in the P301S tauopathy model mouse, which results in motor neuron loss of the spinal cord, there is an approximate 35% decrease in *Slc1a2* expression in disease mice (Hasel et al., 2017). It is unclear if it is reduced EAAT function that leads to initial motor neuron death, or if it is the death of neurons that leads to the decrease in functional EAAT, although a combination of both seems likely to be involved in many cases.

A significant and growing disease burden in modern society is Alzheimer's disease and other dementias. In the UK there are approximately 850,000 people estimated to be living with a form of dementia, with nearly 210,000 new diagnoses each year, overtaking heart disease as the leading cause of death in the UK. Globally, approximately 50 million people are estimated to have dementia, costing the global economy an estimated $818 billion USD each year, with that number expected to reach 82 million people by 2030 at a cost of $2 trillion USD (WHO, 2017, Matthews et al., 2016). Dementias are a collection of neurocognitive disorders that cause varying cognitive dysfunction, with all featuring the presence of neurodegeneration. The most common dementia is Alzheimer's disease (AD) which is characterised by the pathological presence of plaques and neurofibrillary tangles, along with progressive (and often severe) atrophy and neuron loss throughout the hippocampus and cortex.

As with motor neuron disease, there is interest in the role that glutamate dysregulation may play in the progression of neurodegeneration in dementias. From the 1990s it was observed that amyloid β, the main protein found in AD-associated plaques, reduced the function and expression of EAAT1 and EAAT2 in rat hippocampal and cortical astrocytes (Harris et al., 1996, Parpura-Gill et al., 1997, Matos et al., 2008). Studies of human tissue samples have found the aberrant expressions of both the normally astrocyte specific EAAT1 transporter and the enzyme glutamine synthetase in subsets of cortical pyramidal neurons of AD patients, suggesting a marked dysfunction in astrocyte glutamate metabolism (Robinson, 2000, Scott et al., 2002). Reduced expression of both EAAT1 and EAAT2 have further been observed in the hippocampi of patients with AD, alongside a significant decrease in glutamate transporter function in human AD cortices (Jacob et al., 2007, Masliah et al., 1996). Additionally, work with our collaborators has found that there is a significant reduction in both EAAT1 and EAAT2 expression in human frontotemporal dementia patients carrying the *MAPT* exon 10 +16 mutation compared to aged-matched controls (Spires-Jones & Hardingham lab, unpublished). Altogether, accumulated evidence suggests that impaired astrocytic glutamate recycling in the hippocampus and cortex is a feature of dementias and may play a role in the pathological progression of these burgeoning diseases.

1.5 Glutamate transporter regulation

Due to the links of impaired astrocytic glutamate uptake with disease, boosting the function of these transporters to rectify reduced or insufficient glutamate uptake may be a potential therapeutic strategy. In order to do this, one needs to know which signalling pathways are responsible for regulating astrocytic glutamate transporter expression. Unfortunately, these pathways have yet to be fully elucidated, although some hints have appeared. In particular, it has been long known that the presence of neurons is able to increase astrocytic glutamate transporter function.

Before the different EAAT isoforms were isolated, it was observed that culturing cerebellar astrocytes in the presence of cortical neuronal conditioned media increased glutamate uptake (Drejer et al., 1983). Over a decade later, astrocytic EAAT1 and EAAT2 were first found to be significantly downregulated in the striatum following glutamatergic denervation, and the following year it was reported that EAAT1 was upregulated in cortical astrocyte cultures following activation of astrocytic AMPA and kainate receptors (Levy et al., 1995, Gegelashvili et al., 1996). These

reports strongly implicated a role for neurons and neuronally released glutamate in regulating astrocytic glutamate transporters, which was confirmed in 1997 by two independent groups. Swanson and colleagues cultured cortical astrocytes alone or in the presence of cortical neurons, finding that culture with neurons both increased astrocytic EAAT1 expression and robustly induced EAAT2 expression – which was not detected in pure astrocyte cultures (Swanson et al., 1997). It was further documented by Gegelashvili and colleagues that physical culture of neurons with cortical astrocytes increased astrocytic EAAT1 expression, as well as inducing EAAT2 expression (Gegelashvili et al., 1997). Additionally, the group showed that feeding pure cortical astrocytes with neuronal conditioned media was able to induce EAAT2 expression in astrocytes, although they did not find EAAT1 to be affected by conditioned media. They concluded from this work that a neuronally released soluble factor was responsible for the regulation of EAAT2, whereas contact mediated interactions were predominant in the regulation of EAAT1 (Gegelashvili et al., 1997). Interestingly, this group had earlier reported that glutamate was able to increase cortical astrocytic EAAT1 expression, suggesting soluble factors could play a role in regulating this transporter as well (Gegelashvili et al., 1996). Work since then has focused on discovering the signalling molecules and pathways behind this neuronal regulation.

1.5.1 Regulation of astrocytic EAAT1 and EAAT2 by soluble factors

1.5.1.1 Cyclic AMP signalling

One of the first chemicals shown to induce glutamate transporter function in astrocytes was the cyclic AMP analogue, dibutyryl cyclic AMP (db-cAMP) (Hertz et al., 1978). Primary astrocytes grown alone showed little response to glutamate and appeared flat, whilst astrocytes fed with db-cAMP became more morphologically complex with significantly greater glutamate uptake (Hertz et al., 1978, Goldman and Chiu, 1984, Gegelashvili et al., 1996). As a result, many researchers began to treat astrocyte cultures with db-cAMP as standard practice, to make them more reminiscent of their *in vivo* counterparts. This suggests that one potential mechanism for the neuronal regulation of astrocytic glutamate transporters is through an induction of the astrocytic cAMP signalling pathway, which is shown in *Figure 1.4*.

Further evidence for a role of cAMP signalling in astrocytic EAAT regulation comes from studies showing that application of the adenyl cyclase activator forskolin, to stimulate cAMP production,

to both astrocyte cultures and striatal homogenates is able to increase glutamate uptake (Pisano et al., 1996, Schlag et al., 1998). The mechanism behind this cAMP induced upregulation is less clear, with one group finding inhibition of cAMP's downstream target of protein kinase A (PKA) to be sufficient to block the effects of forskolin, whilst others found an effect of PKA inhibition in pure astrocyte cultures but no effect on astrocyte glutamate transporter function when grown in the presence of neurons (Schlag et al., 1998, Pisano et al., 1996). The latter finding suggests that although PKA activation may upregulate EAAT1 and EAAT2 activity in astrocytes in the absence of neurons, in the presence of neurons this pathway is occluded by other mechanisms that are responsible for the observed neuronal regulation of astrocytic EAATs.

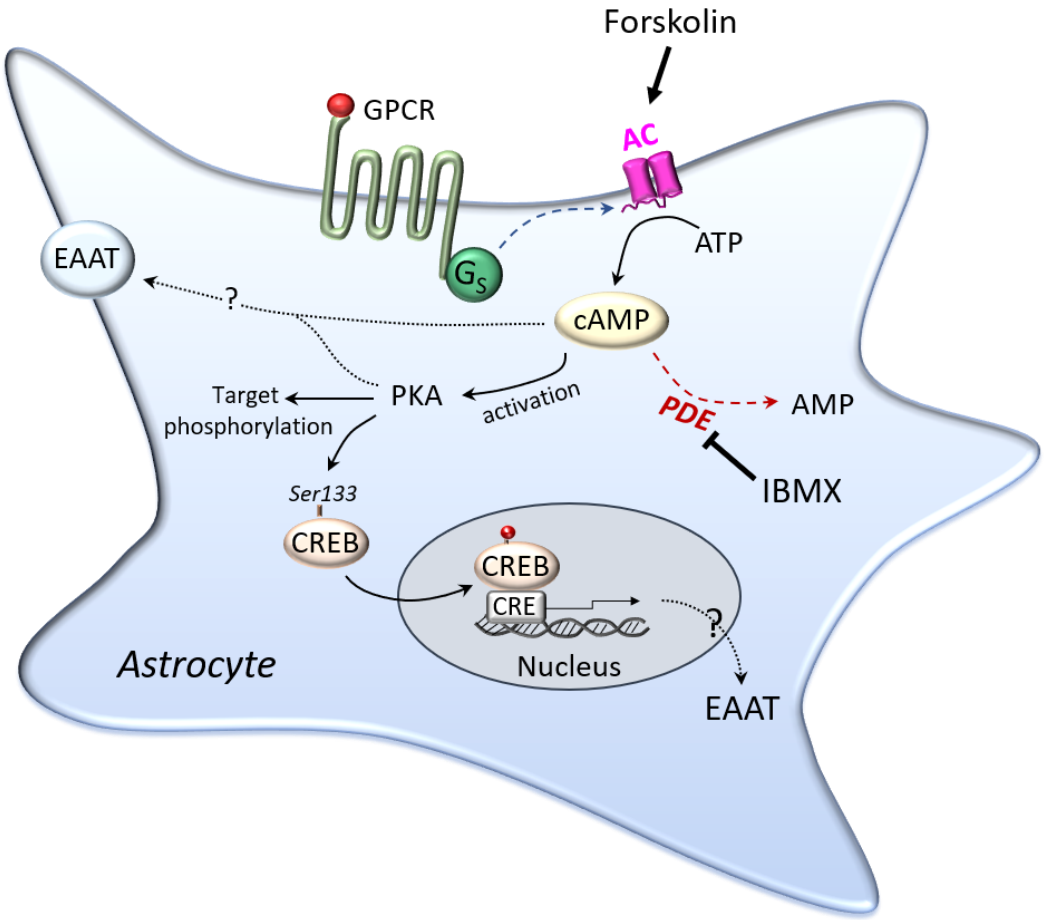


Figure 1.4: The cyclic AMP signalling pathway

Cyclic AMP is produced from ATP following the activation of the adenyl cyclase complex (AD) via Gs proteins. The cAMP activates protein kinase A (PKA), which phosphorylates various targets. This includes the serine133 residue on CREB, with phosphorylation of this residue causing CREB to translocate into the nucleus, activating CRE transcription. cAMP is converted into AMP by phospho-diesterases (PDE).

1.5.1.2 Glutamate signalling

Despite early reporting that astrocytic AMPA and kainate receptor activation may upregulate EAAT expression, and that denervation decreases EAAT2 expression, it is still debatable whether neuronal glutamatergic synaptic activity has a role in astrocytic EAAT expression. Both work from our lab and others have found no effect of pharmacological blockade of neuronal activity on astrocytic EAAT expression (Schlag et al., 1998, Hasel et al., 2017). Contrary to these findings, it has been reported that in hippocampal astrocyte-neuron co-cultures pharmacological block of synaptic activity does reduce protein levels of both EAAT1 and EAAT2 (Perego et al., 2000). Furthermore, acute kainate injections to induce seizure activity in rats were seen to initially cause a significant increase in cortical EAAT2 expression, peaking after 4 hours, before ultimately decreasing below baseline levels (following neuronal death) (Simantov et al., 1999).

1.5.1.3 Other pathways

The EAAT2 transporter is able to be regulated by neuronal secreted factors, and much of the subsequent work into EAAT regulation has been focused on EAAT2 in particular (Gegelashvili et al., 1997). Epidermal growth factor application has been shown to upregulate EAAT2 expression through activation of NF-κB signalling, with neuron-dependent induction of astrocytic NF-κB having been shown to upregulate astrocytic EAAT2 expression (Zelenaia et al., 2000, Sitcheran et al., 2005, Ghosh et al., 2011). Additionally, enhanced expression of Pax6 in pure astrocyte cultures has been recently shown to induce EAAT2 expression, while knockdown of Pax6 in astrocytes grown with neurons was seen to strongly repress neuron-induced EAAT2 expression (Ghosh et al., 2016). However, the authors do not speculate upon the neuronally released factor(s) that may modulate EAAT2 expression through astrocytic Pax6.

1.5.2 Contact dependent regulation of EAAT1 and EAAT2

In contrast to neuronally released factors, relatively little work has investigated the contact-dependent signalling pathways behind neuronal EAAT1 and/or EAAT2 regulation. It has been reasonably well established that unlike EAAT2, neuronal upregulation of EAAT1 expression is via a contact-dependent mechanism and not through a soluble factor, but here the knowledge ends (Gegelashvili et al., 2000). Furthermore, it has not been established if contact-dependent signalling also has a role in EAAT2 regulation.

1.5.2.1 The Notch signalling pathway

Notch is an important contact dependent signalling pathway present in astrocytes; in fact, it is the interaction of Notch ligands expressed on neuronally committed precursor cells with uncommitted precursors that first initiates the precursors' development into astrocyte lineage cells (Namihira et al., 2009). An overview of the Notch signalling pathway is shown in *Figure 1.5*. Briefly, when Notch ligands (for example Delta and Jagged1 & 2) contact the receptors (Notch1-4) the receptors undergo cleavage by the enzyme γ-secretase, releasing the Notch intracellular domain (NICD) of the receptor. The NICD then translocates into the cell nucleus, where it associates with the Notch effector, CBF1, and Mastermind-like (MAML) to activate transcription, with the *Hes* and *Hey* family of genes being well-established examples of this transcriptional pathway (Kopan and Ilagan, 2009, Fischer and Gessler, 2007).

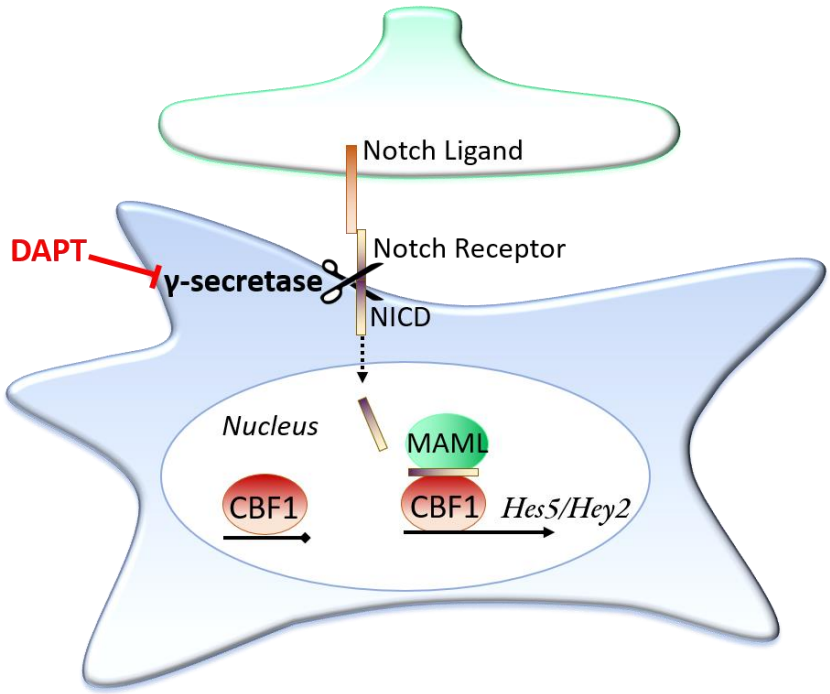


Figure 1.5: The Notch signalling pathway

Notch is a contact dependent signalling pathway. When a Notch ligand contacts a Notch receptor this initiates a cleavage event through the enzyme γ-secretase, releasing the Notch intracellular domain (NICD). The NICD then translocates into the cell nucleus, where it pulls down various proteins, such as MAML, and associates with the Notch effector, CBF1. This association turns on transcription, with *Hes5* and *Hey2* examples of genes transcribed by this cascade. The γ-secretase inhibitor DAPT is able to prevent activation of the Notch signalling pathway as the NICD is unable to be cleaved.

In drosophila only the EAAT1 subtype of high affinity glutamate transporters are found, where it is located on glia cells. Using this model system it was observed that Notch signalling mediated by neuronally expressed Delta ligands induced the expression of EAAT1 in drosophila glia cells (Stacey et al., 2010). If this is a conserved process, these results could suggest a role for Notch signalling not only in allowing astrocyte cell type differentiation, but also in inducing astrocytic EAAT expression. Strengthening the case for Notch, recently, using mouse astrocyte and astrocyte-endothelial co-cultures, endothelial cells were observed to induce EAAT2 expression in astrocytes through contact dependent Notch signalling (Lee et al., 2017). Interestingly, a paper by Angulo-Rojo and colleagues suggests a link between cAMP signalling and Notch in astrocytes (Angulo-Rojo et al., 2013). They observed that application of db-cAMP increased the amount of NICD that translocated into the cell nucleus, and that either application of the γ-secretase inhibitor DAPT to block NICD cleavage, or application of the PKA inhibitor H89 to prevent cAMP mediated PKA signalling, was sufficient to prevent this cAMP induced increase (Angulo-Rojo et al., 2013). They confirmed that db-cAMP was able to induce Notch transcription, first by showing increased CBF1 activity via a luciferase assay. They then demonstrated that db-cAMP treatment increased both *Hes5* gene and Hes5 protein expression, which was also prevented by inhibition of either Notch (via DAPT application) or PKA (via H89) signalling (Angulo-Rojo et al., 2013). This suggests the possibility that the as yet undetermined neuronal signalling molecule that induces astrocytic EAAT expression through the cAMP pathway is in fact physiologically mediated by the contact-dependent Notch pathway in the CNS.

Lending support to the hypothesis that it is neuron-dependent Notch signalling that primarily controls astrocytic EAAT expression, in our lab we have found that neurons are enriched for the Notch ligands, whilst astrocytes are enriched for the Notch receptors (see appendix *A1 i*). Furthermore, we have seen that neurons induce Notch effector activity in astrocytes (appendix *A1 ii*), and that the transcription of the Notch downstream genes is induced in astrocytes by the presence of neurons (appendix *A1 iii*).

In *Chapter 3* I investigate the role of neuronal regulation of astrocytic EAAT expression and function, with a particular aim to answer the outstanding question of whether neuronal Notch signalling is responsible for controlling astrocytic EAAT function.

1.6 Astrocyte-to-neuron communication

After researchers had established that astrocytes respond to neuronal activity, in particular with their roles in glutamate uptake and potassium buffering, the next question was whether astrocytes in turn signalled back to neurons. A passive effect of astrocytes on neuronal function was already implicated due to their control of external K^+ and glutamate concentrations, which could in turn lead to dampening or enhancement of neuronal activity, as well as neuronal death. But the question remained whether astrocytes actively signalled back to neurons, which might additionally give them direct control of either neuronal gene expression and/or function. The first evidence that this could be the case came in the early 1990s, when it was observed that inducing Ca^{2+} events in cultured astrocytes could evoke Ca^{2+} events in neighbouring cultured neurons, via Ca^{2+} induced astrocytic glutamate release (Nedergaard, 1994, Parpura et al., 1994). Additionally, it was observed that, as well as enhancing survivability, cortical neurons grown on cortical astrocytes developed enhanced synchronous Ca^{2+} waves, that were mostly absent in the surviving neurons grown without astrocytes (Nakanishi et al., 1994). It was further shown that glia were also able to induce spontaneous activity in retinal ganglion cell preparations (Pfrieger and Barres, 1997). These early demonstrations of astrocytic effect on neuronal activity triggered an explosion of work over the past two decades investigating the potential signalling events from astrocytes to neurons.

Unfortunately, despite the wealth of evidence that astrocytes exert an influence on neuronal activity, no studies have yet investigated whether and what non-cell-autonomous signalling pathways astrocytes activate in neurons and the functional consequences of this signalling. This is a major gap in our current understanding of the brain, and a question I in part seek to answer in *Chapters 4 & 5* of this thesis, that is: how do astrocytes control neuronal gene expression and function? For the remainder of this chapter I will provide background information on aspects of neuronal function that astrocytes are believed to be actively involved in regulating.

1.7 Astrocytic involvement in neuronal development

A major area of research is focused on the involvement of astrocytes in neuronal development, including their effects on the differentiation of cells into different neuronal subtypes, dendrite and synapse formation.

1.7.1 Morphology and cell type development

Astrocytes have long been observed to affect neuronal morphological development. From 1980 Banker observed that culturing hippocampal neurons with astrocytes significantly increased the number of neuronal protrusions (Banker, 1980). Later it was shown that astrocytes from different regions had different effects on neuronal morphological development. First, dopaminergic neurons from the mesencephalon were either plated onto astrocytes prepared from the mesencephalon or striatal regions. The dopaminergic neurons grown on astrocytes from the same region developed multiple complex branches, whereas those grown on astrocytes from the striatum developed relatively few branches (Denis-Donini et al., 1984). Later the group investigated growing striatal or mesencephalic neurons on either striatal or mesencephalic astrocytes: again, neurons from the mesencephalon showed more mature development when grown on astrocytes also from the mesencephalon, whereas striatal neurons appeared more mature when grown on striatal astrocytes rather than mesencephalic (Autillo-Touati et al., 1988, Chamak et al., 1987). This work indicated that astrocytes not only effect neuronal morphology, but that astrocytes from different regions are able to exert different control over neurons.

Multiple studies have since shown a role for astrocytes in guiding axon and dendritic growth as well as determining neuronal morphology throughout different brain regions, including the brainstem, cortex, hippocampus, retina, olfactory bulb, midbrain and striatum (Martin et al., 2012, Cheng et al., 2018, Ebrahimi et al., 2016, Le Roux and Esquenazi, 2002, Jacobs et al., 2016, Steinbach et al., 2001, Bailey et al., 1999, Garcia-Abreu et al., 2000, Wagner et al., 1999, Gates and Dunnett, 2001, Matsutani and Yamamoto, 1998). Both physical location and orientation of astrocytes as well as secretion of astrocytic factors, such as fibroblast growth factor, are believed to be important for this control of neuronal morphology (Chotard and Salecker, 2004, Procko and Shaham, 2010, Le Roux and Esquenazi, 2002).

1.7.2 Synaptogenesis

The fundamental feature of CNS function is synaptic communication between neurons, mediating information transfer and storage. In order for successful neurotransmission to occur, pre- and post-synapse formations must develop on the right neurons at the right physical location, and correctly localise in apposition to each other. Furthermore, each synapse must form a correct specialisation,

for example becoming a GABAergic or glutamatergic synapse. The process of synaptogenesis is therefore both complex given the variety of neurotransmitter receptors and tightly regulated, with the underlying mechanisms yet to be largely understood (for review see Kurshan & Shen, 2019 and Südhof, 2018). In general, pre-synaptic densities form at the end of axons, where they contain neurotransmitter vesicles and release machinery, while post-synaptic densities form (typically) on dendritic spines and contain neurotransmitter receptors. The two densities co-localise in apposition to each other, although whether it is the pre-synapse that induces the formation of the post-synaptic density, or the post-synapse that guides the pre-synapse towards it may vary depending on region and synapse. Additionally, the process of specialisation may be hardwired into certain cell types, whereas for others specialisation may instead occur after pre- and post-synapse co-localisation (Südhof, 2018, Kurshan and Shen, 2019).

Several proteins have been shown to be important in synapse formation, chiefly among them the cell adhesion molecules (CAMs) which are found in the pre- and post-synapses and are believed to be important in synaptic formation and positioning. Many CAMs have been described, including neuroligins, neurexins, latrophilins, pentraxins and brain angiogenesis inhibitors (BAIs) (Südhof, 2017, Südhof, 2018). The importance of these proteins has been demonstrated with hippocampal neurons grown with HEK or COS cells engineered to express different CAMs. Neuroligin-1 and -2 expression in HEK cells were shown to induce pre-synapse formation in axons, as was synCAM (with co-expression of synCAM and glutamate receptors in HEK cells sufficient to form functional post-synaptic responses), whilst neurexin expressing COS cells caused both GABA and glutamatergic post-synapse formation on neuronal dendrites (Graf et al., 2004, Biederer et al., 2002, Scheiffele et al., 2000). Aside from neuronally expressed CAMs, several other players have been implicated in synapse formation, including a variety of astrocyte associated proteins.

Suspicions that astrocytes may be involved in neuronal synaptogenesis were first roused by work from the Barres group during the development of purified retinal ganglion cell (RGC) cultures. They initially observed that cultured RGCs showed little spontaneous activity on their own (after 20 days in culture only 63% of RGCs displayed spontaneous activity), whereas growing these cells in the presence of glia from the superior colliculus increased the number of RGCs displaying spontaneous activity to 100% (Pfrieger and Barres, 1997). Interestingly, the authors mention that co-culturing RGCs with purified *neurons* from the superior colliculus had a similar effect to glia in

inducing spontaneous synaptic activity, which would suggest the effect is not specific to glia, but rather a general effect of cells from that brain region on RGCs. However, they did not explore this idea further and instead showed that culturing purified RGCs in the presence of glia conditioned media was sufficient to replicate the effect of direct co-culture on spontaneous events (Pfrieger and Barres, 1997). Next, they demonstrated that this increase in spontaneous events was not necessarily due to an increase in excitability in co-cultured RGCs but instead due to an increase in synapse number, as there was a 12-fold increase in frequency (and amplitude) of miniature EPSCs (mEPSC) in co-cultured RGCs. I note here that they did not investigate whether there was an increase in excitability in co-cultured RGCs in addition to the observed increase in quantal release probability, as increased excitability could also cause an increase in spontaneous activity. Using electron microscopy (EM) they found that mono-cultured RGCs developed structurally normal synapses, although there were twice as many synapses in the presence of glia, which was further corroborated by the presence of positive pre-synaptic puncta staining in purified RGCs. From this the authors conclude that glia must secrete factors that transform structurally present but silent synapses in RGCs into functionally active synapses (Pfrieger and Barres, 1997).

Several years later the original authors largely repeated their original findings: increased spontaneous activity in RGCs cultured either in the presence of glia from the superior colliculus or glial conditioned media, whilst still observing structural synapses in the absence of glia (Ullian et al., 2001). They confirmed it was a protein in the glial conditioned media, as treatment with proteinase prevented the effect (Nägler et al., 2001). Additionally, they found that although there were similar amounts of pre- and post-synaptic associated protein, there was an increase in “puncta” like formations of these proteins in co-cultured RGCs, with approximately a 5-fold increase in co-localised pre- and post-synaptic puncta in RGCs grown in the presence of astrocytes. They explored the effect of astrocytes on synaptic stability by first growing RGCs in the presence of astrocyte conditioned media (ACM) for 5 days before exchanging the ACM for unconditioned media for a further 6 days. Removal of ACM reduced both the pre-synaptic quantal content of evoked activity as well as co-localised synaptic puncta to approximately a third of the values recorded in the presence of ACM (Ullian et al., 2001). They then investigated the developmental time point that synaptic puncta appear in the superior colliculus of rats (where RGCs extend their axons to), finding that puncta appeared during the first post-natal week, which corresponded to the time when

astrocytes appeared to undergo rapid proliferation as assessed by S100β staining (Ullian et al., 2001). They hypothesised that astrocytes are required for the formation of stable synapses, which is why synapses don't appear until after astrocyte proliferation. Disappointingly, the authors did not investigate the effect of co-culture of RGCs with neurons from the superior colliculus on synapse co-localisation and stability, noting that they observed these neurons to have a similar effect as glia on the spontaneous activity of RGCs in their earlier work.

Nevertheless, following on from this work a series of astrocyte factors have now been implicated in synaptogenesis, an overview of which is given in *Figure 1.6*. In this introduction I will specifically focus on work related to astrocyte involvement in excitatory, that is glutamatergic, synapse formation, although it should be noted that astrocytes may also have a role in GABAergic synapse formation.

Astrocyte protein	Neuronal target	Function	Locations shown (species)
Thrombospondin (TSP-1 & TSP-2)	α2δ-1 receptor	Increases the number of structural but silent synapses	Cultured RGCs (rat) Cortex & SC (mouse) Cortex following injury (mouse)
Hevin (SPARCL1)	Neuroligins 1-3 & neurexin-1α	Bridge between post-synaptic NL1-3 & pre-synaptic NRX1α proteins to stabilise excitatory thalamocortical synapses	Cultured RGCs (rat) Primary visual cortex, SC (mouse)
Sparc	Unknown	Opposes the action of hevin (i.e. removing Sparc increases synapse number)	Cultured RGCs (rat) SC (mouse)
Glypican (GP-4 & GP-6)	RPTPδ & RPTPσ	Promotes structural synapse formation & induces post-synaptic GluA1 receptor subunit clustering	Cultured RGCs (rat) SC, hippocampus, visual cortex (mouse)
Cholesterol	Unknown	Increases spontaneous activity	Cultured RGCs (rat)
Neuroligin-2	Unknown	Promotes functional excitatory synapse formation	Visual cortex (mouse)
Chordin-like 1	Unknown (not BMP receptors/ signalling)	Increases GluA2 subunit expression in excitatory synapses	Cultured RGCs (rat) Cortex (mouse)

Figure 1.6 Astrocytic proteins associated with excitatory synaptogenesis

A list of astrocyte secreted factors (bar neuroligin-2) that are proposed to be involved in excitatory synaptogenesis. Abbreviations: receptor protein tyrosine phosphatases (RPTP), superior colliculus (SC), retinal ganglion cells (RGCs). Sources: (Allen et al., 2012, Blanco-Suarez et al., 2018, Farhy-Tselnicker et al., 2017, Kucukdereli et al., 2011, Eroglu et al., 2009, Stogsdill et al., 2017, Risher et al., 2018, Christopherson et al., 2005, Lau et al., 2017, Mauch et al., 2001).

1.7.2.1 Thrombospondins

Thrombospondins (TSPs) are a group of oligomeric, multidomain, extracellular Ca^{2+} binding glycoproteins, that interact with many components, including the extracellular matrix (ECM), cell surfaces, cytokines and growth factors, to regulate a multitude of activities throughout the body (Adams and Lawler, 2011). There are five members of the family in vertebrates, TSP1-5, which fall into two subgroups according to their structural assembly: group A (TSP-1 and -2) that form trimers, and group B (TSP3-5) that assemble as pentamers (Adams and Lawler, 2011). In the brain TSP1-4 gene expression has been observed across microglia, oligodendrocytes, neurons and astrocytes, although their cell-type expression pattern differs between mice and humans (Zhang et al., 2014).

In 2001, TSP-1 and -2 were first proposed as astrocytic secreted factors necessary for the promotion of excitatory synapse formation in RGCs. (Christopherson et al., 2005). The authors demonstrated that either a cortical astrocyte feeder layer or ACM was able to increase the number of co-localised synaptic puncta in purified rat RGCs, however only the feeder layer was able to increase mEPSC events in these retinal cells (Christopherson et al., 2005). They went on to show a potential role for both TSP-1 and -2 in the increase of puncta co-localisation, with application of TSP-1 to RGCs increasing puncta, and depletion of TSP-2 from ACM reducing the number of co-localised puncta, although there was an increase in non-co-localised puncta in TSP-2 depleted ACM (Christopherson et al., 2005). As with ACM treatment, neither TSP-1 or -2 were able to increase the spontaneous activity of RGCs, unlike direct co-culture with astrocytes. However, ACM, TSP-1 and TSP-2 were all demonstrated to increase the pre-synaptic activity of RGCs, whilst having no effect on mEPSC frequency or amplitude, or response to glutamate application, compared to controls (Christopherson et al., 2005).

With regards to TSP expression, they observed widespread immunoreactivity to TSP-1/2 throughout the superior colliculus and cortex in the P8 rat brain, which had all but disappeared by P21. They showed that some synaptotagmin (a pre-synaptic vesicular membrane docking protein) immunoreactivity appeared co-localised with TSP-1/2 immunoreactivity at P8 – but given the fact essentially all P8 tissue showed immunoreactivity to TSP-1/2, any protein tested would be expected to show apparent co-localisation with TSP-1/2. As TSP-1/2 was absent by P21, they conclude

TSP-1/2 is only transiently needed to stabilise structural but silent synapses. As their earlier work found that removal of astrocytes resulted in reversal of puncta co-localisation this would mean that some other astrocytic factor is required for the ongoing puncta stability at later time points (Ullian et al., 2001, Christopherson et al., 2005). Using specific TSP-1 and TSP-2 antibodies they detected both TSP-1 and TSP-2 protein in P5 rat cortical tissue, that was largely gone in adult brain samples. Using RT-qPCR they detected mRNA of both TSP-1 and TSP-2 in purified cortical astrocytes – but they either did not investigate or disclose whether they detected TSP-1 or -2 protein in purified astrocytes.

To investigate the *in vivo* influence of TSP-1 and -2 they investigated the number of pre-synaptic puncta in cortical tissue from TSP-1 and TSP-2 knockout mice. There was no difference in cortical pre-synaptic puncta number between wild-type mice and TSP-1 or TSP-2 knockout animals. They then created a TSP-1/TSP-2 double knock out mouse. In this model they observed a 31% reduction in co-localised puncta in the cortex of P21 animals (Christopherson et al., 2005). They reported no difference in dendritic area between wild-type and double knock-out animals but did not directly compare whole cell morphology between animals. Perhaps the biggest limitation of this study is the lack of tissue let alone cell-type specificity: they employed a global double TSP-1/2 knockout model – given the abundance and multifaceted roles of TSPs throughout the body and brain, one might expect some differences in global knockouts (Adams and Lawler, 2011, Risher and Eroglu, 2012). To date, no astrocyte conditional TSP-1 or -2 knock-out has been generated, which would greatly strengthen the case for astrocyte TSP-1/2 involvement in synaptogenesis.

Several years later the same group determined that the synapse promoting effect of TSPs was mediated by their interaction with the neuronal α2δ-1 subunit (also known as the gabapentin receptor), a subunit of L-type voltage-gated Ca^{2+} channels. Alpha-2δ-1 is found throughout the brain, with particularly strong expression in the cortex, hippocampus and hypothalamus, and has a role in Ca^{2+} channel regulation (Cole et al., 2005). The authors first determined that the effect of TSPs on inducing excitatory synaptic puncta in RGCs was mediated by the EGF-like repeat domain of TSP complexes (Eroglu et al., 2009). They then investigated the presence of proteins that expressed domains that the EGF domains of TSPs are known to interact with, which included the α2δ group, finding that RGCs expressed the α2δ-1 subunit. They further observed that in the rat cortex α2δ-1 subunits appeared in puncta formations in apposition to both pre- and post-synaptic

puncta, and using immunoprecipitation confirmed that the EGF domains of TSPs were associated with α2δ-1. Following overexpression of α2δ-1 in RGCs they found that the TSP induced increases in synaptic puncta were enhanced. Furthermore, knockdown of α2δ-1 in RGCs prevented both astrocyte and TSP mediated increases in synaptic puncta, whilst treatment with the α2δ-1 antagonist gabapentin prevented the effects of ACM and TSP on RGC puncta number (Eroglu et al., 2009).

Turning to an *in vivo* model they first overexpressed α2δ-1 in mouse cortical cells and measured the number of co-localised synapses at P21. They found a nearly 2-fold increase in co-localised vGlut2 and PSD-95, but no difference in co-localised vGlut1 and PSD-95. Conversely, treatment of pups with gabapentin for 6 days resulted in a significant decrease in co-localised vGlut2/PSD-95 puncta in P7 mice cortices (Eroglu et al., 2009). Interestingly, several years later using an α2δ-1 knockout mouse model they observed the opposite: a reduction in vGlut1/PSD-95 puncta in cortical layer II/III cells, but no change in vGlut2/PSD-95 co-localisation (Risher et al., 2018). Correspondingly, they observed a significant reduction (-58%) in mEPSC frequency of these neurons, as well as a slight reduction in amplitude, as would be expected from reduced synapse numbers. In contrast to the previously claimed lack of morphological difference of cortical neurons in TSP-1/TSP-2 double knockout mice, this time they investigated morphology and found a significant difference between cells in the α2δ-1 knockout model and wild-type controls, with knockout animals showing a reduced dendritic length and significantly less branching (Risher et al., 2018). This discrepancy could be due to the other roles α2δ-1 plays besides its role as a TSP receptor. Using serial section EM they investigated the effect of α2δ-1 on spine morphology, finding that in mutant animals there was a significant reduction in the number of spines. As α2δ-1 is expressed throughout the body, they generated a conditional α2δ-1 model which sparsely removed α2δ-1 from cortical neurons. As with global knockouts, there was a reduction in both synapses and spine numbers in this conditional model (Risher et al., 2018).

Separately, a group investigating the protective mechanisms of gabapentin following cortical insult likewise used an α2δ-1 knockout model. In the insult model they employed, there was an increase in TSP and excitatory synaptogenesis as well as cell death following injury in wild-type mice, which was prevented by either gabapentin treatment or by α2δ-1 knockout (Lau et al., 2017). Whilst α2δ-1 knockout prevented an increase in synapses following injury, there was no difference in co-localised

vGlut1/PSD-95 staining in cortical cells between wild-type control, wild-type plus gabapentin treatment or α2δ-1 knockout animals that had not undergone cortical insult (Lau et al., 2017). Correspondingly, they found no difference in mEPSC frequency between these conditions, along with observing no difference in phenotype, behaviour or epileptogenic potential between wild-type and α2δ-1 knockout animals (Lau et al., 2017). These findings conflict with the other report, suggesting that astrocytic TSP activation of α2δ-1 is not needed for normal neuronal synapse development, and instead the pro-synaptic effects of TSP/α2δ-1 signalling only occur following a pathological insult.

Overall, these results demonstrate that TSP signalling through α2δ-1 can promote excitatory synaptogenesis. However, several questions still remain, firstly whether it is astrocyte secreted TSPs that mediate this response *in vivo,* and secondly whether this signalling pathway is required for normal excitatory development, or if it only comes into play *in vivo* under pathological conditions.

1.7.2.2 Hevin/SPARCL1 and Sparc

Hevin, also known as SPARC-like 1 (SPARCL1), is a secreted matricellular protein (as are thrombospondins), found in both astrocytes and neurons throughout the brain (Mongrédien et al., 2019). Given the observed effect of TSPs on synaptogenesis, the Barres/Eroglu group investigated whether other astrocyte secreted proteins of the same family may also have an effect on synaptogenesis (Kucukdereli et al., 2011). They found that hevin and sparc were both enriched in astrocytes, but unlike TSPs, they were enriched throughout adult life. As standard, to begin they grew purified RGC cultures, which were either left alone or treated with ACM, hevin, or TSP, finding that like ACM or TSP treatment, hevin significantly increased the number of co-localised puncta in RGCs (Kucukdereli et al., 2011). Again, as with TSP, hevin increased the number of structural but silent synapses in RGCs, as measured with EM and functionally by lack of change in mEPSC frequency. Depletion of hevin from ACM reduced the number of synapses by 30%, with hevin depletion of TSP-1/2 knockout ACM completely preventing any effect of ACM on synapse number. Of note, in their earlier study depletion of ACM TSP-2 alone was sufficient to block the entire effect of ACM on RGC synapse number, yet in this later study ACM lacking both TSP-1 and TSP-2 only partially reduces the effect of ACM (Christopherson et al., 2005, Kucukdereli et al., 2011).

Conversely, application of sparc to RGCs did not have any effect on synapse number, but when applied in combination with hevin reduced the effect of hevin application, suggesting sparc may work in opposition to hevin. Indeed, when the authors depleted sparc from ACM they saw an increase in synapse number, agreeing with this hypothesis (Kucukdereli et al., 2011). Sparc was not found to co-immunoprecipitate with hevin, suggesting the two proteins may competitively interact with hevin's synapse inducing neuronal target. To investigate the role of these proteins *in vivo* the authors investigated the expression of hevin and sparc in the superior colliculus (SC) of mice, where RGC axons terminate. Both hevin and sparc were widely expressed in the SC by P14, and both appeared to be predominantly astrocytic, and found in association with each other and with glutamatergic synapses (Kucukdereli et al., 2011). In hevin knockout mice they observed a 30% reduction in excitatory synapse number in the SC, whereas in sparc knockouts they found a 70% increase in synapse numbers, further suggesting these two proteins have oppositional functions to each other (Kucukdereli et al., 2011).

In a later paper the group explored the role of hevin in the cortex (Risher et al., 2014). As with the SC, they found extensive hevin staining that peaked in the cortex between P14-25. In the synaptic zone of the primary visual cortex in hevin knockout animals they found a reduction in thalamocortical vGlut2/PSD-95 co-localised puncta that was not due to a decrease in thalamic projections, and unexpectedly, an *increase* in intracortical vGlut1/PSD-95 co-localised puncta (Risher et al., 2014). Overall, they saw no difference in mEPSC frequency of pyramidal cortical cells, with the suggestion that the increase in vGlut1/PSD-95 is a compensation for the lack of vGlut2/PSD-95 thalamocortical synapses in hevin knockouts. They then investigated the effects of hevin on spine morphology, finding that hevin knockouts had an increase in immature filapodial protrusions and a significant reduction in mature mushroom spines in the cortex of adult mice. Moreover, they noted an increased occurrence of projections synapsing onto the dendritic shaft, rather than onto spines, as well as an increase in spines with multiple excitatory contacts, normally a feature restricted to immature synapses (Risher et al., 2014). Looking further into spines with multiple contacts they observed that the majority of these cases involved contacts from both intracortical and thalamocortical inputs in hevin knockout mice, with intracortical inputs eventually overcoming thalamocortical inputs, suggesting that hevin is required for stabilising thalamocortical synaptic inputs (Risher et al., 2014).

Having discovered a potential role for astrocyte secreted hevin in thalamocortical synapse stabilisation (noting the use of global hevin knockout mice, rather than astrocyte conditional knockouts) in their next paper they propose a mechanism for this hevin mediated control (Singh et al., 2016). Due to their observations that hevin appeared to modulate both pre-synapses as well as post-synaptic NMDA GluN2B subunits they hypothesised that hevin may play a role in linking pre- and post-synapses together. To test this hypothesis they first cultured RGCs in the presence of HEK293 cells expressing either membrane anchored hevin or membrane anchored control proteins (mCherry or alkaline phosphatase), and found increased puncta localisation of the pre-synaptic marker synapsin-1 around HEK cells expressing hevin (Singh et al., 2016). However, cleavage of hevin between its N- and C- domains prevented pre-synaptic clustering. To investigate the effects on the post-synapse, they co-cultured RGCS in the presence of magnetic beads coated in either full length, N-terminal domain or C-terminal domain hevin, finding either full length or the C-terminal domain was able to induce clustering of the post-synaptic marker homer-1. Unlike culturing RGCs in the presence of full length hevin, application of either hevin's C-terminal, N-terminal, or a combination of both was unable to increase synaptic puncta co-localisation of RGCs (Singh et al., 2016). This demonstrates that although specific terminals are involved in the localisation of pre- or post-synaptic puncta, the full length of unbroken hevin is required for co-localisation of synapses, suggesting hevin acts as a bridge.

As mentioned in *Chapter 1.7.2*, the neuroligin and neurexin CAMs are able to organise co-localisation of pre- and post-synapses, with post-synaptically expressed neuroligins attracting post-synapses, and pre-synaptically expressed neurexins attracting post-synapses. Given this, the group hypothesised that hevin may act in conjunction with a subset of these CAMs to facilitate this synaptic organisation. They found that hevin was associated with neuroligins 1-3 (NL1-3), and that its effect on enhancing puncta co-localisation in RGCs was dependent on the expression of NL1-3 in RGCs (Singh et al., 2016). Furthermore, NL1 was co-immunoprecipitated with the C-terminal domain of hevin, suggesting that the observed interaction of the C-terminal domain with the post-synapse is via interaction with NL. They next looked into the pre-synaptic neurexins and found that hevin was only significantly co-immunoprecipitated with the neurexin isoform NRX1α, and that this association did not occur with the C-terminal domain of hevin. Under normal conditions NRX1α is able to directly bind to various neuroligin isoforms including NL1 and NL2, but is

unable to interact with NL1 variants containing a B insertion (NL1B) (Chih et al., 2006, Boucard et al., 2005). Unlike NRX1α, the authors demonstrated that hevin was able to bind to the NL1B variant, which could indicate hevin's physiological role as a mediator of NRX1α and NL1B binding (Singh et al., 2016). *In vivo*, both NRX1α and NL1 knockout mice were found to have a significant decrease in vGlut2/PSD-95 co-localisation and a corresponding increase in vGlut1/PSD-95 co-localisation in the primary visual cortex, as they had likewise observed in hevin knockout mice (Singh et al., 2016, Risher et al., 2014). However, these knockout models also prevent the binding of NL1 and NRX1α to all the direct binding partners that they have on neurons, which would be expected to have pronounced effects on synapse formation, independent of any interactions with astrocytic proteins. On the other hand, knocking out NRX1α from specifically thalamic neurons, or NL1 specifically from cortical neurons impaired hevin's ability to increase co-localisation of vGlut2/PSD-95 puncta. Although this work does not show that hevin is specifically required for NRX1α and NL1B binding, it does suggest a role for hevin in facilitating NRX1α and NL interactions in thalamocortical synaptogenesis.

They further investigated the requirements of astrocytic hevin for ocular dominance plasticity in mice, hypothesising that reduced synaptic NMDA receptor subunit expression in hevin knockouts would impair plasticity. Indeed, they found an impairment in this form of plasticity in hevin knockout animals, which was rescued by viral re-expression of hevin in astrocytes. Slightly misleadingly they claim astrocytic hevin is therefore needed for ocular dominance plasticity: they did not show the effects of neuronal, or non-astrocytic, hevin re-expression, and a more convincing demonstration would have been via generation of a conditional astrocytic hevin knockout model. Evidence strongly shows a role for hevin in synaptic regulation, but the extent of this role in neuronal synaptic development, and whether hevin secretion is a specific to astrocytes, is yet to be determined.

1.7.2.3 Glypicans

While both thrombospondins and hevin promote structurally sound but post-synaptically silent excitatory synapses, the heparan sulphate proteoglycans glypican-4 and -6 (GP-4 & -6), are hypothesised to transform silent synapses into active synapses. This was first demonstrated in RGCs, with Allen and colleagues showing that co-culturing RGCs with a feeder layer of astrocytes

increased mEPSC frequency, as well as increasing the surface expression and puncta formation of three AMPA receptor subunits, GluA1, GluA2 and GluA4, without altering these proteins' total tissue expression (Allen et al., 2012). Despite the group's repeated earlier observations that only direct co-culture is sufficient to induce an increase in mEPSC frequency with ACM treatment having no effect on mEPSC frequency in RGCs (see Pfrieger & Barres, 1997 and Christopherson et al., 2005), this time they saw that ACM treatment not only increased mEPSC frequency in RGCs, but that the effect was stronger than co-culture with astrocytes (Allen et al., 2012). They analysed the ACM to determine what proteins were present, and by fractionation narrowed the list of candidate proteins down to 25, which they expressed individually in COS7 cells, eventually finding that GP-4 was able to increase mEPSC frequency in RGCs in the presence of TSP. A western blot for GP-4 on media derived from RGCs or astrocytes showed that astrocytes not RGCs secrete GP-4. Application of purified GP-4 to mono-cultured RGCs in the absence of TSP was found to be sufficient to increase both mEPSC frequency and amplitude, with GP-4 treatment found to specifically upregulate the surface expression of the GluA1 AMPA receptor subunit (Allen et al., 2012). Additionally, GP-4 treatment of RGCs increased co-localised synaptic puncta by nearly 3-fold, which was nearly as much as astrocyte co-culture.

Along with GP-4, the related glypican family member GP-6 was also amongst the 25 candidate proteins they had isolated, and so they investigated whether GP-6 had a similar effect. Like GP-4, GP-6 was able to induce structural synapses in RGCs and increase surface expression of GluA1 subunits. Depletion of both GP-4 and GP-6 from ACM prevented the ACM increases in mEPSC amplitude and GluA1 surface clustering, indicating that GP-4 and GP-6 are involved in strengthening the post-synapse. However, depletion of GP-4 and GP-6 did not prevent their observed increase in mEPSC frequency, and there was still a significant increase in co-localised synaptic puncta in this GP depleted ACM. These results seem somewhat incongruous: GP-4 is able to increase frequency of mEPSC events and synaptic puncta, yet the depletion of both GP-4 and GP-6 from ACM has no effect on mEPSC frequency or synaptic puncta. Mechanistically, the authors showed that GP first clusters GluA1 on the RGC process surface, with formation of the post-synaptic machinery following sometime after this clustering. Furthermore, removal of GluA1 by siRNA prevented GP-4 mediated increases in synapse number, whereas ACM treatment of GluA1 lacking RGCs still induced synapse formation, suggesting that the TSP and GP synapse

formation pathways are different (which only raises further questions as to why GP depleted ACM had no effect on total synapse number) (Allen et al., 2012).

Turning to an *in vivo* model, they found that GP-4 and GP-6 were 3 to 6 times more enriched in astrocytes than neurons, respectively, in the cortex, and 1.5 to 2 times more enriched in the hippocampus at P7. However, it is worth noting that by P12 in the hippocampus GP-4 expression was largely seen in neurons and reduced in astrocytes, and by P24 entirely restricted to pyramidal neurons (Allen et al., 2012). Expressional change of GP-4 with age in the cortex, or GP-6 in cortex or hippocampus, was not shown or described. In a GP-4 knockout mouse they found mEPSC amplitudes of hippocampal CA1 neurons to be reduced by 22% in P12 pups, although this difference had largely gone by P24. Structurally, there was no decrease in co-localised synaptic puncta in GP-4 knockout mice at P12, but there was a significant reduction in co-localised puncta containing GluA1 (Allen et al., 2012).

Recently, the group reported on the signalling pathway through which GP-4 recruits GluA1 AMPA receptor subunits (Farhy-Tselnicker et al., 2017). As GP-4 led to an increase in surface clustering of GluA1 subunits, they theorised that this might be in part due to an increase in AMPA receptor stability. Glypicans are known to interact with both the neuronally expressed type 2a receptor protein tyrosine phosphatases (RPTPs) and leucine-rich repeat transmembrane proteins (LRRTMs), with several studies showing the binding of GPs, and in particular GP-4, to LRRTMs to be important in excitatory synapse regulation (Coles et al., 2011, de Wit et al., 2013, Siddiqui et al., 2013). Secondly, several factors are involved in regulating AMPA receptor strength and stability, including the extracellular acting neuronal pentraxins NP1 and NP2 (also known as NARP) (Bassani et al., 2013). As such, Allen and colleagues investigated the relation between GP-4 and neuronal pentraxins, and RPTPs/LRRTMs.

To begin, they undertook gene expression profiling of purified cultured RGCs which had been treated with either GP-4 or TSP for 12 hours, finding no overlap in gene expression changes between these two conditions (Farhy-Tselnicker et al., 2017). Amongst the 49 genes whose expression changed in the presence of GP-4 was NP1, a known regulator of AMPA receptors, with GP-4 treatment of RGCs additionally shown to increase extracellular NP1 concentrations by nearly 2-fold (Farhy-Tselnicker et al., 2017). Treating RGCs with either ACM or GP-4 resulted in an

increase in NP1 accumulation on neuronal surfaces, whereas TSP-1 had no effect on NP1, and this accumulation still occurred even in the presence of an mRNA synthesis inhibitor. Confirming NP1 interaction with GluA1 is necessary for GP-4 increases in synapse number, when GP-4 was applied while inhibiting NP1's ability to interact with GluA1 there was no longer an induction of co-localised synaptic puncta (Farhy-Tselnicker et al., 2017). Similarly, knockdown of NP1 with siRNA prevented GP-4 mediated increases in both surface GluA1 clusters and synaptic puncta. Next they determined what protein GP-4 interacted with to cause this increase in NP1 secretion, finding that the axonally expressed RPTPδ and RPTPσ subtypes were responsible for GP-4's synaptogenic and GluA1 clustering effect (Farhy-Tselnicker et al., 2017).

To determine the importance of this interaction *in vivo*, they investigated the localisation of GP-4 in the superior colliculus and visual cortex in P6 mice, which is an interesting choice as their previous paper focused on the hippocampus due to GP-4's prominent expression in that region. They claim GP-4 is localised to astrocytes in the cortex and not neurons, although in the image they provide I noted apparently equally strong GP-4 co-expression with neuronal Tubb3 (Farhy-Tselnicker et al., 2017). In GP-4 knockout mice they observed a significant increase in NP1/vGlut2 co-localised puncta, suggestive of a failure to release NP1 from pre-synaptic terminals. To investigate if it is astrocyte GP-4 that is responsible for inducing NP1 release they generated a GP-4/aldh1l1cre line to remove GP-4 from astrocytes. Unfortunately staining in the cortex showed a large proportion of neurons to be also expressing aldh1l1cre, so they focused their investigation on the superior colliculus, which showed a somewhat lower neuronal expression of aldhl1lcre, although still quite significant (Farhy-Tselnicker et al., 2017). Regardless, they observed a significant increase in NP1 pre-synaptic puncta in the conditional knockout line compared to controls in the superior colliculus (Farhy-Tselnicker et al., 2017). Knocking out either GP-4 or RPTPδ resulted in a significant reduction in co-localised synaptic puncta in the superior colliculus, as well as a significant reduction in post-synaptic GluA1 clustering (Farhy-Tselnicker et al., 2017).

Overall, it appears GP-4 can act on the pre-synaptic RPTP receptors to increase NP1 release, which increases GluA1 clustering. More work needs to be done to answer the question of the relative astrocytic importance in this mechanism in the wider CNS, and what the functional outcomes may be.

1.7.2.4 Cholesterol, Neuroligins & Chordin-like 1

Several other astrocyte proteins have been implicated in excitatory synaptogenesis, including cholesterol, neuroligins and chordin like-1. Early on in the hunt for astrocyte derived factors that were mediating an increase in activity of RGCs cholesterol was proposed as a candidate (Mauch et al., 2001). Pfrieger and colleagues found an induction of apolipoprotein E (apoE) spots in RGCs treated with ACM, and although apoE application did not increase synaptic activity, application of cholesterol, which can be carried by apoE-containing lipoproteins, was able to increase spontaneous activity in RGCs (Mauch et al., 2001). Additionally, they found depletion of cholesterol from ACM was able to prevent the ACM induced increase in spontaneous activity in RGCs, and that application of cholesterol increased the number of co-localised synaptic puncta. From this they concluded that astrocyte derived cholesterol, carried by apoE complexes, was the astrocyte derived factor increasing spontaneous activity and synapse numbers in RGCs (Mauch et al., 2001). No further reports have documented evidence of astrocyte derived cholesterol as being important for synaptogenesis, nor investigated whether it may play a role beyond RGCs, or if it has a role *in vivo*. Interestingly, apoE/apoE receptor signalling is important for correct brain organisation and synaptic plasticity, with apoE being a primarily astrocyte produced factor (Holtzman et al., 2012, Lane-Donovan and Herz, 2017).

More recently, astrocytic neuroligins were demonstrated to promote excitatory synapse formation in neurons. This work is promising, as neuroligins are CAMs with known roles in synapse formation when expressed in neurons. After finding that neuroligins 1-3 (NL1-3) expressed on astrocytes are important for astrocytic morphological elaboration, Eroglu and colleagues noted that in the visual cortex of mice that had had NL2 sparsely knocked down, there were approximately half the number of co-localised excitatory synapses in the region of NL2-free astrocytes compared to the regions of neighbouring wild-type astrocytes (Stogsdill et al., 2017). Functionally, in a conditional astrocyte NL2 knockout mouse line, mEPSC amplitude and frequency in layer V visual cortical neurons were significantly less compared to heterozygous littermates (Stogsdill et al., 2017). It should be noted the authors employed a GLAST-CreERT2 line to remove astrocytic NL2 but did not report on the cell-type expression of this transgene in the region of interest. This is significant, as GLAST-CreERT2 lines have been shown to lack specificity for astrocytes; indeed, in

the hippocampus a higher proportion of neurons express the GLAST-CreERT2 than astrocytes (Srinivasan et al., 2016).

Lastly, astrocyte secreted chordin-like 1 (Chdl1) has been proposed as necessary for the transformation of excitatory synapses into mature GluA2 AMPA receptor subunit containing forms (Blanco-Suarez et al., 2018). AMPA receptors lacking GluA2 are Ca^{2+} permeable, and their expression is associated with synapse immaturity, whereas most AMPA receptors in the mature cortex contain the GluA2 subunit, rendering them Ca^{2+} impermeable (Brill and Huguenard, 2008). After proposing that GP-4 and GP-6 are able to induce GluA1 clustering, Allen and colleagues hypothesised that another astrocyte secreted factor subsequently induced the recruitment of the GluA2 subunit. As with previous studies, the group began by preparing purified RGC cultures, showing that RGCs treated with ACM had higher GluA2 clustering (Blanco-Suarez et al., 2018). They then ran a biochemical screen for candidate proteins in ACM, with the candidate Chrdl1 being shown to induce GluA2 clusters on RGCs. They also found that treating RGCs with a physiological level of Chdl1 was able to increase co-localised synapse numbers to the same extent as co-culture with astrocytes, as well as being sufficient to increase mEPSC frequency (Blanco-Suarez et al., 2018). This is an interesting finding, given that depletion of other factors, such as TSP, GP-4, GP-6, or cholesterol from ACM were able to prevent increases in synapse number in RGCs: their findings here suggest the Chrdl1 present in those previous studies should have at least partially masked those results. It is also a further confliction with previous work as to whether ACM is sufficient to increase mEPSCs, or whether direct astrocyte co-culture is required for this induction. Regardless, the group generated a Chrdl1 knockout animal, finding a significant reduction in GluA2 positive excitatory synapses in the cortex, along with a significant reduction in excitatory thalamocortical synapses, but no change in excitatory intracortical synapses (Blanco-Suarez et al., 2018). Unlike RGCs, in these cortical cells they observed no difference in mEPSC frequency in Chrdl1 knockout animals, although there was a slight increase in mEPSC decay time, which they attribute to a decrease in GluA2 subunit expression (Blanco-Suarez et al., 2018).

1.7.2.5 Retinal Ganglion Cells

Common to most of the studies investigating astrocyte involvement in excitatory synaptogenesis is the use of RGCs as a model. This was initially brought about as purified neurons typically do not

survive very well in culture without astrocytes present, making it very hard to elucidate the impact of astrocytes on their development. However, in the 1980s Barres developed a protocol for the isolation and growth of RGCs in culture (Barres et al., 1988, Barres and Chun, 1993). These cells were capable of surviving without astrocytes when they were grown in a defined medium, opening the door to investigate the effects of glia on synaptogenesis in retinal cells. Although these cells are not necessarily representative of neural cells from other areas of the brain, they have since become the model from which generalisations of astrocyte-neuron interactions are derived. Here I will briefly summarise what these cells are, and the limitations of using this system as a model for these investigations.

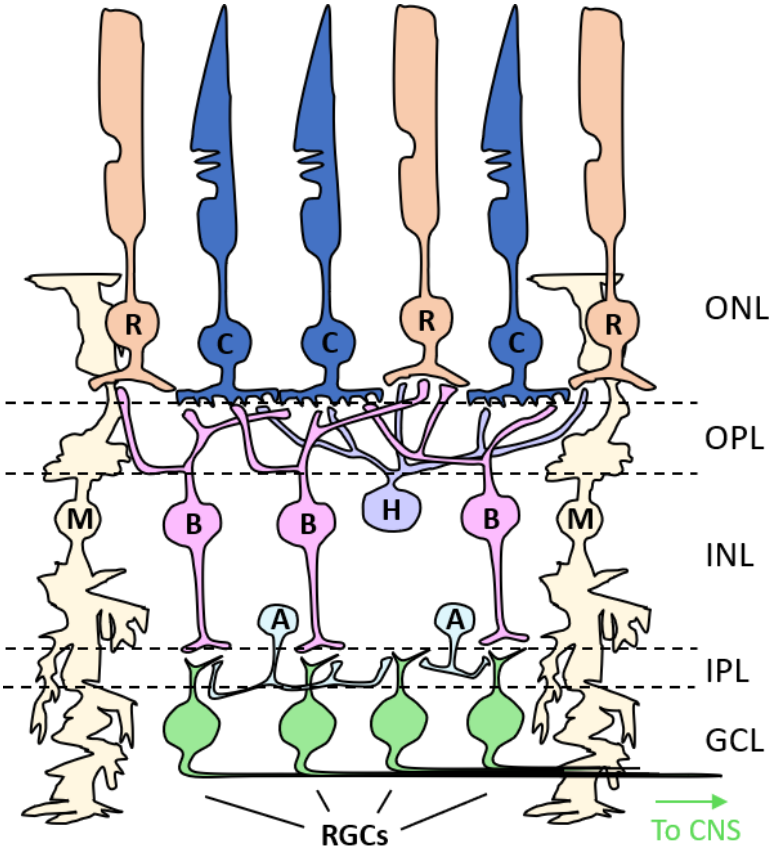


Figure 1.7 Retinal cell network

Retinal ganglion cells (RGCs) receive input from amacrine (A) and bipolar cells (B), which receive light input from rod (R) and cone (C) photoreceptors. RGCs project axons through the optic nerve into the CNS, where they form synapses in visual centres of the brain. Surrounding the neural cells within the retina are numerous Müller glia cells (M), which span the retinal layers. ONL = outer nuclear layer, OPL = outer plexiform layer, INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer, H = horizontal cell. References: (Masland, 2012, Bringmann et al., 2006, Hoon et al., 2014).

Retinal ganglion cells are a heterogeneous family of cells located in the retina that are solely responsible for transmitting the visual information received by the eye into the brain (*Figure 1.7)*. They receive input from retinal amacrine and bipolar cells, which they send into the CNS through axons that project into the lateral geniculate nucleus within the thalamus or the superior colliculus within the midbrain (Masland, 2012, Cruz-Martín et al., 2014, Reinhard et al., 2019). Currently there are estimated to be around 40 distinct types of RGCs, which receive and transmit different visual information, including the newly described intrinsically photosensitive RGCs, which express melanopsin, allowing them to directly respond to light input (Bae et al., 2018, Baden et al., 2016, Rheaume et al., 2018, Schmidt and Kofuji, 2011). Surrounding these RGCs are Müller glia, the predominant, if not sole, macroglia within the retina, where they fulfil the role of astrocytes (Bringmann et al., 2006). Both Müller glia and RGCs (along with other retinal cells) are produced from retinal progenitor cells, and not the neural radial glia progenitor cells of the brain from which astrocytes and other CNS neurons are derived (Cepko, 2014).

This presents some obvious limitations when using purified RGCs as a model for synaptogenesis in general. Firstly, RGCs are a highly organised heterogeneous group of cells that are only just beginning to be characterised, for example the subset of RGCs that respond directly to light could result in environmental light having an unknown impact on recorded activity. Secondly, despite surviving in culture alone, they are not a resilient group of cells: their survival is dependent upon feeding with an extensive list of hormones and factors, importantly requiring frequent supplementation with insulin, brain derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and forskolin (Meyer-Franke et al., 1995). Thirdly, they are not derived from the same precursors as neurons of the CNS – they are a distinct lineage surrounded by other cells from this same lineage such as Müller glia, and hence it is hard to extrapolate findings on their development to the development of neurons and astrocytes that are derived from a separate pool. For example, synapse development between retinal cells is highly dependent on light input, which is not true for the brain at large. Finally, and perhaps most importantly, within the retina the dendrites of each RGC form selective synapses with specific bipolar or amacrine cells, and transmit this received information through terminals that synapse onto highly specific neurons within the CNS – they do not form synapses with each other (Gollisch and Meister, 2010). This renders the use of purified RGCs as a model for synaptogenesis questionable, as synapse formation would *not*

normally occur between these cells. This problem is highlighted by the early finding that culturing RGCs with neurons from the RGC projection area, the superior colliculus, was sufficient to induce synaptogenesis in the absence of glia (Pfrieger and Barres, 1997). Therefore, the lack of synapse formation seen between isolated RGCs in culture does not necessarily represent a deficiency in these cells' intrinsic synaptogenic capabilities. It may instead be physiologically normal, as they have no target cells present, and the forced astrocytic induction of synapse formation between RGCs may in fact be the abnormal situation.

Given these problems it is desirable to develop a system for studying the effects of astrocytes on neurons that uses cells from other areas of the brain, such as the cortex, to understand the true relationship between astrocytes and their regional neuronal partners. Despite the historical finding that neurons do not typically survive well without astrocytes, a better understanding of factors needed for neuronal survival has meant that today primary CNS neurons are capable of surviving without glia, given the right media and culture conditions. For example, in our lab we are able to consistently produce healthy viable primary cultures of pure cortical neurons free of glia, by combination of an optimised culture preparation procedure, and supplementing basic feeding media with a combination of rat serum, B-27 and glutamine. Perhaps a key feature of our system is the density of the neurons, as we plate cells at a high, more physiologically accurate, concentration of ~500,000 cells per cm^2. Even halving this density results in a decrease in neuronal health and survival of our cells, and yet it is typical to read papers that plate at much lower densities still, from 20,000-100,000 cells per cm^2. As we are able to produce robust cortical neuronal cultures that do not require astrocytes, this presents an excellent opportunity to address whether or not these findings in RGCs are generalisable to cortical neurons, and to further elucidate the influence of astrocytes on neuronal functional development in a neuronal population beyond RGCs.

1.8 Astrocyte involvement in neuronal activity

Astrocytes play an active role in modulating neuronal activity by releasing various molecules which neurons are able to sense and/or uptake. A common feature of the work in RGCs presented in *Chapter 1.7* was the increase in spontaneous activity driven by astrocytes. Although part of this may be due to an increase in synapse formation in RGCs, there are other ways in which astrocytes can modulate neuronal activity. For example, astrocytes may play a passive role in supporting neuronal

activity by providing neurons with neurotransmitter and metabolic precursors, which becomes particularly important under conditions of high activity. Beyond passive modulation, astrocytes are now suspected to have important roles in regulating neuronal plasticity, for example they are able to release neuro-modulatory molecules, such as D-serine and glutamate, which can potentiate neuronal receptor activation. Here I will briefly discuss some of the ways in which astrocytes are proposed to control neuronal plasticity.

1.8.1 Synaptic plasticity

Synaptic plasticity refers to changes in neuronal responses that results in an increase or decrease of synaptic activity that persists over time. There are several types of plasticity, including long-term potentiation (LTP) and long-term depression (LTD) which are thought to mediate learning and memory, as well as homeostatic plasticity that seeks to oppose pathological changes in activity in order to maintain stable neuronal activity (Martin et al., 2000, Tien and Kerschensteiner, 2018). All forms of plasticity are integral features of cognitive function, and recently astrocytes have been shown to play an active part in these neuronal phenomena.

1.8.1.1 Long-term potentiation

Long-term potentiation refers to a long term increase in synaptic strength, induced following repetitive high frequency stimulation or pre-synaptic activity coincident with post-synaptic depolarisation, and is typically dependent on NMDA receptor activation (Martin et al., 2000, Bliss and Collingridge, 1993). Several studies have now reported a requirement of astrocyte secreted D-serine, an NMDA receptor co-agonist, for the induction of LTP. Early after the turn of the millennia a potential dependence on astrocytes for NMDA receptor dependent LTP was discovered in hippocampal culture preparations (Yang et al., 2003). The authors prepared hippocampal cultures grown either on a bed of astrocytes or without astrocytes but instead fed with ACM to ensure synaptogenesis. They were able to induce LTP only in co-cultured hippocampal neurons, not those just fed with ACM, and further showed that the induction of this LTP was dependent on the NMDA receptor co-agonist D-serine (Yang et al., 2003). In acute hippocampal slices they additionally showed that either degrading endogenous D-serine or inhibiting the NMDA receptor co-agonist binding site, caused a significant reduction in induced LTP. They found that application of 0.2 mM glutamate to glial, but not neuronal, terminal preparations resulted in an increase in

D-serine concentrations (Yang et al., 2003). This work demonstrated that this form of LTP is not mediated by a passively released astrocytic factor, but instead that astrocytes sense neuronal activity and actively respond.

Several years later the importance of astrocytic released D-serine for LTP was further described (Henneberger et al., 2010). The authors recorded field potentials in the hippocampal *stratum radiatum*, inducing LTP by Schaffer collateral stimulation, in the vicinity of a patch-clamped astrocyte. Under normal conditions they were able to induce LTP in the vicinity of recorded astrocytes, which was unaffected by addition of exogenous D-serine and blocked by antagonism of the NMDA receptor. However, when they clamped the astrocyte's internal Ca^{2+} concentrations they were unable to induce LTP in the astrocyte's vicinity, but applying exogenous D-serine was able to rescue LTP (Henneberger et al., 2010). To confirm the inhibition of LTP in the area of Ca^{2+} clamped astrocytes was due to inhibition of astrocytic D-serine release they included a serine racemase inhibitor to the internal patch solution to prevent astrocytic D-serine production and ran a high frequency stimulation protocol in the presence of an NMDA receptor antagonist, AP-V, to exhaust astrocytic D-serine stores before washing out the AP-V. When they ran the LTP induction protocol, now with no astrocytic D-serine stores, they were unable to induce LTP (Henneberger et al., 2010). These findings suggest that neuronal transmitter release induces Ca^{2+} signalling in astrocytes, which causes the release of the NMDA receptor co-agonist D-serine, which is required for the successful induction of NMDA receptor dependent LTP.

Later on in 2012 the involvement of astrocytes in LTP was first demonstrated *in vivo* in the cholinergic system (Navarrete et al., 2012). The authors induced LTP of CA3-CA1 hippocampal synapses in anaesthetised rats by tail-pinch sensory stimulation, noting that this stimulation also induced Ca^{2+} elevations in astrocytes. LTP was prevented by administration of either muscarinic receptor (mAChR) or metabotropic glutamate receptor (mGluR) antagonists, although astrocytic Ca^{2+} responses persisted in the presence of mGluR antagonism (Navarrete et al., 2012). The authors used inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice ($IP_3R2^{-/-}$), which primarily mediates intracellular astrocytic Ca^{2+} signalling, to investigate whether astrocytic Ca^{2+} elevations were necessary for this form of LTP (Navarrete et al., 2012). Application of acetylcholine to these slices induced Ca^{2+} elevations in neurons from knockouts and wild-types, but only induced Ca^{2+} elevations in wild-type astrocytes. There was a significant reduction in LTP induced by sensory

stimulation in these knockout animals, suggesting activity mediated release of astrocytic glutamate through Ca^{2+} signalling is required for cholinergic LTP *in vivo* (Navarrete et al., 2012) .

More recently, astrocyte stimulation was shown to be sufficient to increase memory in *in vivo* behaving mice (Adamsky et al., 2018). The authors expressed the Gq-coupled designer receptor hM_3Dq in hippocampal CA1 astrocytes, allowing them to induce Ca^{2+} elevations in these CA1 astrocytes by applying the drug CNO. To begin, they found that activation of these designer receptors in CA1 astrocytes caused a significant increase in both frequency and amplitude of mEPSC events in CA1 neurons in slice. When they induced LTP via Schaffer collateral stimulation they found that the activation of astrocytic Gq receptors to increase astrocytic Ca^{2+} resulted in a 50% enhancement of LTP (Adamsky et al., 2018). This enhancement was masked by treatment with D-serine and blocked by chelation of astrocytic Ca^{2+}. Behaviourally, they found that mice expressing the designer Gq receptor in hippocampal astrocytes that had been treated with CNO had enhanced performance in contextual memory tasks, with a 40% increase in recall in a contextual freezing foot-shock task compared to controls (Adamsky et al., 2018).

1.8.1.2 Long-term depression

Unlike LTP, long-term depression is marked by a persistent decrease in synaptic strength, often following extended low-frequency stimulation, and like LTP is thought to be involved in learning and memory (Collingridge et al., 2010). LTD is not as well described as LTP, but astrocytic involvement in LTD is now an emerging area of research, with several reports beginning to implicate these cells mechanistically.

A form of LTD termed spike-timing dependent LTD (t-LTD) occurs when pre-synaptic activity follows post-synaptic spiking within a few milliseconds, and is believed to be important for sensory mapping in the developing cortex (Min and Nevian, 2012, Dan and Poo, 2006). This form of plasticity requires activation of both pre-synaptic NMDA receptors as well as cannabinoid receptors (CB_1R) (Min and Nevian, 2012). In 2012 Min and Nevian showed that astrocytic Ca^{2+} signalling was induced by activation of astrocytic CB_1R, and that this activation was present and required for the induction of t-LTD in the rat barrel cortex (Min and Nevian, 2012). They then demonstrated that astrocytic CB_1R-induced Ca^{2+} signalling caused the release of astrocytic glutamate, and that this glutamate was required for the induction of t-LTD in the barrel cortex.

Furthermore, they found that stimulating an astrocyte whilst concurrently stimulating pre-synaptic afferents was sufficient to induce t-LTD (Min and Nevian, 2012).

More recently, astrocytes were shown to be involved in hippocampal NMDA receptor dependent LTD induced by low-frequency stimulation. The authors first found that low-frequency stimulation was able to induce astrocytic Ca^{2+} responses in hippocampal slices, causing astrocyte release of glutamate, which then activated neuronal NMDA receptors (Navarrete et al., 2019). When they quenched internal astrocytic Ca^{2+}, or recorded from slices of $IP_2R2^{-/-}$ mice, they observed strong impairments of low-frequency stimulation induced NMDA receptor dependent LTD compared to controls. To investigate the role of astrocytic released glutamate in LTD, they created mice lines lacking astrocytic expression of VAMP2 and VAMP3 to prevent astrocytic exocytic glutamate release, with hippocampal cultures from these mice showing a marked reduction in LTD compared to control (Navarrete et al., 2019). The authors then used mice expressing channel-rhodopsin 2 in astrocytes, finding that optogenetic stimulation of astrocytes increased astrocytic Ca^{2+} and induced astrocytic glutamate release, and that this was able to induce NMDA receptor dependent LTD (Navarrete et al., 2019). As previous studies had implicated a role for p38 mitogen-activated protein kinases (MAPK) the group then investigated the role of astrocytic versus neuronal p38 MAPK activity in LTD (Navarrete et al., 2019). They found that astrocytic p38α MAPK, but not neuronal, was specifically required for the induction of LTD, and that astrocytes lacking p38α MAPK no longer released glutamate following low-frequency stimulation (Navarrete et al., 2019). Behaviourally, they found that mice lacking astrocytic p38α MAPK had a significant enhancement in long-term memory as assessed by a contextual fear task compared to both controls and mice lacking neuronal p38α MAPK (Navarrete et al., 2019).

These works clearly implicate a central role for activity induced release of astrocytic glutamate in the development of various forms of LTD.

1.8.1.3 Activity-regulated homeostatic plasticity

Homeostatic plasticity of neuronal activity is the attempt to maintain synaptic activity around an optimal set-point: in response to too much activity, changes will occur to dampen the neurons' activity levels down, whilst too little activity induces changes to neuronal properties to try and increase activity levels back up. There are two main aspects to this plasticity, the modulation of a

cell's intrinsic properties to increase or decrease excitability, and the modulation of synaptic strength which can be measured by changes in mEPSC frequency and amplitude (Lazarevic et al., 2013, Turrigiano et al., 1998, Echegoyen et al., 2007, Desai et al., 1999). Homeostatic plasticity is believed to involve the regulation of numerous channels and the mechanisms behind its different facets are only just beginning to be elucidated (Lazarevic et al., 2013, Lee et al., 2015).

Here too in this fundamental process astrocytes may play vital roles. In 2006 Stellwagen and Malenka discovered that astrocytic TNF-α was required for the homeostatic increase in mEPSC amplitude following 48 hours of activity deprivation in hippocampal pyramidal cells (Stellwagen and Malenka, 2006). Separately, it was shown that hippocampal astrocytes *in situ* respond to long-term inhibition of neuronal activity with increased Ca^{2+} signals mediated by group I mGluRs, whilst long-term increases in neuronal firing led to a decrease in astrocytic mGluR mediated spontaneous Ca^{2+} events (Xie et al., 2012). This demonstrated that astrocytes are able to sense and respond to long-term dysregulation of neuronal activity, a necessary requirement for mediating homeostatic responses. To date no other work has explored the involvement of astrocytes in either homeostatic regulation of excitability or whether they are required for reducing synaptic strength down in hyperactive scenarios, although their involvement in homeostatic processes has been hypothesised (Thalhammer and Cingolani, 2014, Sims et al., 2015).

1.8.2 Neuronal K_{IR} channels

The potassium inward rectifiers (K_{IR}) are a family of K^+ channels which are typically open under resting membrane potentials allowing the flux of K^+ ions. Upon membrane depolarisations their pores become increasingly blocked, preventing the efflux of K^+ ions, and giving them their characteristic inward rectification. Each K_{IR} channel is comprised of four K_{IR} subunits resulting in a heteromeric channel (Bichet et al., 2003). The subunits fall into 7 subfamilies (K_{IR}1-7), with channels typically formed by different subunits within the same family, although that is not always the case (Bichet et al., 2003). The different K_{IR} channels fall into four functional groups, constitutively active (K_{IR}2.X), G-protein gated (K_{IR}3.X), ATP-sensitive (K_{IR}6.X) and transport channels (K_{IR}1.1, 4.X, 5.X & 7.X) (Hibino et al., 2010). The subunits are found throughout the brain, and within the cortex the K_{IR}2.X and K_{IR}3.X subunits are predominantly expressed (Shcherbatyy et al., 2015). K_{IR} channels play many physiological roles, including the control of

neuronal excitability through alterations in membrane properties, with K_{IR} channels leading to decreased membrane resistance and hyperpolarised membrane potentials (Hibino et al., 2010). Thus, higher K_{IR} channel expression decreases excitability whilst lower expression leads to an increase in excitability. A clear example of this is seen in astrocytes: their abundant expression of $K_{IR}4.1$ is what sets their exceedingly low membrane resistance and hyperpolarised membrane potentials (Butt and Kalsi, 2006, Neusch et al., 2006).

Given K_{IR}'s involvement in setting neuronal excitability it is possible that the regulation of their expression may be one mechanism underlying the homeostatic plasticity of neuronal excitability. In support of this, K_{IR} expression was found to be increased in epileptic tissue, and chronic electroconvulsive shock was found to significantly upregulate both $K_{IR}3.1$ and $K_{IR}3.2$ subunits (Young et al., 2009, Stegen et al., 2009, Pei et al., 1999, Stegen et al., 2011). This could suggest that in response to pathologically high activity levels these channels undergo homeostatic upregulation in an attempt to mitigate elevated activity levels. Despite the protective role K_{IR} channel upregulation may play in response to pathologically high excitability, too much K_{IR} can conversely be a bad thing. Several patients with severe developmental delays effecting language, social and motor skills (with one patient still unable to walk at the age of 17) were found to have a gain-of-function mutation in $K_{IR}6.2$ that meant the channel was always open (Gloyn et al., 2004). There were no other abnormalities in neurological structure or reductions in cortical or cerebellar size, and no abnormality in muscle biopsies, in these patients that could otherwise explain the severe neurological phenotype.

Given the functional importance of K_{IR} in controlling neuronal activity, and the severe effects when this goes wrong, it is important to understand how these channels are regulated. One intriguing possibility is that astrocytes might control neuronal K_{IR} expression. Firstly, astrocytes are known to increase neuronal activity, and K_{IR} expression is important for setting cell excitability. As such, one-way astrocytes might increase neuronal activity could be by decreasing neuronal K_{IR} expression. Interestingly, cholesterol has been shown to regulate K_{IR} function, decreasing $K_{IR}2.1$ and $K_{IR}3.1$ activity, with hypercholesterolemia causing a significant downregulation of $K_{IR}3.1$ expression in aortic smooth muscle cells (Ya-Jun et al., 2016, Rosenhouse-Dantsker, 2019). As mentioned earlier, astrocyte-secreted cholesterol was proposed as a mediator of synaptogenesis due its effect on increasing spontaneous neuronal activity: maybe this increase in activity was due to an increase in

excitability borne from K_{IR} downregulation (Mauch et al., 2001). Secondly, astrocytes have a suspected role in regulating activity-dependent homeostasis, whilst K_{IR} channels are suspected to be subject to activity-regulated homeostasis. Another possibility then, is that astrocytes may somehow control the expression of neuronal K_{IR} in an activity-dependent manner.

In *Chapters 4 & 5* I investigate whether astrocytes control neuronal K_{IR} expression, and whether this leads to functional alterations in neuronal activity.

1.9 Thesis summary

In this thesis I aim to elucidate some of the key interactions between neurons and astrocytes and the outcomes of this interaction on CNS function. My thesis is broken down into two broad sections, first looking at the influence of neurons on astrocytes, and second, looking at the effect of astrocytes on neurons.

In the first results chapter (*Chapter 3*) I seek to answer whether neurons control astrocytic EAAT function, and to find the signalling pathway mediating this control. To do this I used our labs published mixed species astrocyte/astrocyte-neuron RNA-sequencing dataset to investigate neuronal control over astrocytic glutamate transporters. I confirm that neurons increase astrocytic glutamate transporter expression and function, as previous studies have likewise shown. I then use information gained from work in our lab to find the signalling pathway from neurons to astrocytes that controls the regulation of these astrocytic transporters. I demonstrate that neuronal contact-dependent Notch signalling controls astrocytic EAAT expression and function. I further show that this Notch signalling is constantly required in order to maintain astrocytic EAAT expression, which may have important consequences for neurodegenerative disease.

In the second part of my thesis I seek to answer how cortical astrocytes control cortical neuronal gene expression and function, which is split across two chapters (*Chapter 4* and *Chapter 5*). In the beginning of *Chapter 4* I used our labs cortical culture setup to address the open question of how astrocytes control cortical neuronal gene expression. To date, there are no reports on any neuronal genes being regulated by astrocytes, primarily due to the historical problems of being unable to maintain neurons in the absence of astrocytes. Our lab used a robust system for growing purified

cortical neurons in the absence of glia, on which an RNA-sequencing screen of cortical rat neurons grown alone or in the presence of cortical mouse astrocytes was run to address this gap in our knowledge. This dataset, presented for the first time in this thesis, reveals that the cortical neuronal transcriptome is extensively under the control of non-cell-autonomous signalling from astrocytes. I focus on a functionally important group of genes that were uncovered by this screen as being downregulated in neurons by astrocytes: the K_{IR} family of channels.

I first ask how astrocytic regulation of K_{IR} controls neuronal membrane properties and excitability, finding that reduced K_{IR} expression in cortical neurons due to astrocytes results in an expected alteration in membrane properties, leading to an increase in excitability. I then show that the expression of these K_{IR} channels is under activity-dependent homeostatic regulation by the second week of culture, but only in the presence of astrocytes. Next, I seek to uncover the signalling pathway behind the reduction of cortical neuronal K_{IR}, finding that it is an astrocyte secreted factor that reduces K_{IR} expression. Lastly in *Chapter 4,* I present the results of my proteomic screen of ACM, providing a list of candidate molecules for the astrocyte-regulation of K_{IR} expression which will be the focus of experiments going forward.

In *Chapter 5* I ask what the possible role of astrocytes, and astrocyte control of neuronal K_{IR}, is on excitatory synaptic development and activity in cortical neurons. I find that cortical neurons grown in the presence of astrocytes have significantly higher spontaneous activity than those grown without, and that K_{IR} overexpression in co-cultured neurons blocks spontaneous activity. This suggested that astrocytic enhancement of cortical neuron excitability due to the downregulation of neuronal K_{IR} led to an increase in neuronal activity. I then investigate whether this enhanced activity might, in part, drive the previously reported astrocyte mediated excitatory synaptogenesis. Unexpectedly, I discover that cortical neurons grown in the absence of astrocytes form functionally active synapses to the same extent as their counterparts grown with astrocytes under basal conditions – suggesting that not only does K_{IR} expression not control cortical developmental synaptogenesis, but neither do astrocytes! However, following 48 hours of activity deprivation, which is known to cause a homeostatic increase in synaptic strength, only cortical neurons grown in the presence of astrocytes were observed to have a significant increase in mEPSC frequency. Moreover, following TTX washout of activity-deprived neurons grown in the absence of astrocytes

there was very little induced spontaneous network events, whereas TTX removal from neurons grown with astrocytes induced robust spontaneous bursts of network activity.

Based on my findings I suggest that astrocytes play a fundamental role in controlling activity-dependent homeostatic plasticity, both at the level of excitability and synaptic strength, with this dual control leading to a dramatic regulation of neuronal activity.

1.9.1 Aims

In my thesis I seek to address the following four primary aims:

1. To establish that neurons induce a functional increase in cortical astrocytes' capacity to transport glutamate, and to determine whether this non-cell-autonomous induction is mediated by the contact-dependent Notch signalling pathway.

2. To establish whether and to what extent there is a non-cell-autonomous signalling pathway from astrocytes to cortical neurons.

3. To determine whether non-cell-autonomous signalling from cortical astrocytes to neurons leads to functional changes in neurons, in particular whether this signalling leads to an increase in neuronal activity.

4. To investigate whether any observed changes induced in neurons via astrocyte-signalling is upstream to the induction of excitatory synaptogenesis that has been reported to occur via other laboratories.

Chapter 2

Materials and Methods

Chapter 2 – Materials and Methods

2.1 Cell culture: cortical tissue

Primary cell cultures of cortical neurons and cortical astrocytes were generated from tissue collected from embryonic day 17.5 CD1 mice and embryonic day 20.5 Sprague-Dawley rats. Embryos were euthanized by decapitation, their brains removed, and cortices dissected out into dissociation media (DM+K: 81.8 mM Na_2SO_4, 30 mM K_2SO_4, 5.84 mM $MgCl_2$, 252 μM $CaCl_2$, 1 mM HEPES, 0.1% Phenol Red, 20 mM glucose and 1 mM kyurenic acid). Cortices were placed in round bottom culture tubes (Corning) and digested in DM+K containing 20 units/mL of L-cysteine-activated papain enzyme (Worthington Biochemical Corporation) for 40 minutes at 37°C, shaking every 10 minutes, with fresh papain/DM+K solution added halfway through.

Following digestion cortices were washed twice with DM+K, and twice with 1% NBA media, containing Neurobasal-A (Gibco), Anti-Anti (anti-bacterial/antimycotic, Invitrogen), B27 supplement (Life Technologies), glutamine (1 mM, Sigma) and 1% rat serum (Envigo). Cortices were then placed in a fresh 2 mL of 1% NBA and triturated gently using a 5 mL serological pipette on a low speed pipette gun (approximately 50 times). A further 2 mL of 1% NBA was then added to the tube, and after letting the tissue settle for a minute, the top 2 mL of suspension was collected and placed in a fresh tube. The remaining suspension was again triturated. If no tissue chunks remained it was added to the collected suspension, if not then a further 2 mL of 1% NBA was added, and the process repeated until all cells were completely dissociated. The dissociated suspension was then topped up to 10 mL with 1% NBA, and added to Opti-MEM (Gibco) solution supplemented with glucose (20 mM, Sigma) and Anti-Anti, at a concentration of 1 rat cortex/14 mL solution, and 1 mouse cortex/7 mL solution (giving an approximate concentration of 1,000,000 cells/mL).

For neuronal cultures, 0.5 mL cell suspension (500,000 cells/well) was plated down onto glass coverslips (VWR) that had been placed into the wells of 24-well plates (Greiner), and pre-coated with poly-d-lysine (5 μg/mL, Sigma) and Laminin (13 μg/mL, Roche). For co-culture experiments, the coverslips had been pre-seeded with astrocytes 72 hours prior. The plates were then incubated at 37°C with 5% CO_2 for two hours, before aspirating the Opti-MEM solution, and feeding with 1 mL 1% NBA containing 4.8 μM cytosine arabinoside (AraC) (unless otherwise stated) for cells in

Chapter 4 and *5*. Cells used in *Chapter 3* were instead fed with 0% NBA (NBA media without the addition of rat serum added). On day in vitro (DIV) 4 the wells were topped up with an additional 1 mL of fresh feeding media.

For astrocyte cultures, 10 mL of cell suspension (~130,000 cells per cm^2) was pipetted into a 75 cm^2 cell culture flask (Greiner) pre-coated with poly-d-lysine (5 μg/mL, Sigma). The plates were incubated at 37 °C with 5% CO_2 for two hours, before aspirating the Opti-MEM solution, and feeding with 20 mL of DMEM solution (Dulbecco's Modified Eagle Medium with high glucose, L-glutamine, sodium pyruvate and phenol-red, Gibco) that was supplemented with Anti-Anti and 10% fetal bovine serum (Gibco). On DIV4, 10 mL of DMEM was removed and a fresh 10 mL added.

To generate pure astrocytic cultures the flasks were passaged on DIV7 and DIV11. Briefly, cells were washed with phosphate buffered solution (PBS, Gibco), and then incubated in 5 mL of 0.05% trypsin-EDTA (Gibco) at 37 °C for 5 minutes. The flasks were then gently rocked to ensure all cells were detached from the flask, collected into a 15 mL falcon tube with 5 mL of DMEM, and centrifuged at 800 RPM for 4 minutes. The supernatant was aspirated, and the cells were dissociated and collected into fresh DMEM at a concentration of 1x 75 cm^2 flask of cells into 50 mL media. This suspension was then divided between three new poly-d-lysine coated 75 cm^2 flasks (15 mL per plate). On DIV11 the process was repeated, but instead plating the astrocyte suspension onto coated coverslip-containing 24-well plates at a concentration of 1 mL/well (at a cell density of ~100,000 astrocytes per coverslip). Nota bene, as astrocytes are proliferative cells, the final density of astrocytes per coverslip at the time of recording will be greater than the density at plate down.

On (neuronal) DIV7 a 50% media exchange was done to feed the cells. For cells that were to be grown to DIV15, on DIV9, 11 and 13/14, 50% media exchanges were carried out using 0% NBA media supplemented with 10 mM glucose. Cells grown to DIV21+ followed the same feeding schedule, except on DIV13/14 a 50% media exchange was done with transfection media (TMITS) containing (in mM): 114 NaCl, 5.3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 1 glycine, 30 glucose, 0.5 sodium pyruvate, 0.2% $NaHCO_3$, 0.001% phenol red, 10% Minimum Essential Media (-glutamine, Gibco), 1% Anti-Anti and 1% insulin-transferrin-selenium (100x, Invitrogen). Fifty percent media exchanges with TMITS were repeated on DIV17 and DIV21.

2.2 Cell culture: retinal ganglion cells

Preparation of retinal ganglion cells (RGC) was done following the Cold Spring Harbor Laboratory protocol (Winzeler and Wang, 2013), with minor adjustments.

Postnatal day 5-6 rat pups were euthanized by decapitation before the eyeballs were enucleated into chilled DM+K media and kept on ice. Retinas were dissected from the eye in chilled DM+K, by first making an incision into the front of the eye, removing the lens and vitreous matter and then carefully tearing the sclera away from the retina. Retinas were kept in ice cold DM+K until all dissections were finished, before being transferred to a round bottomed culture tube containing DM+K with 20 units/mL of L-cysteine-activated papain enzyme and deoxyribonuclease I (DNAse; 0.07%, Worthington) and incubated for 30 minutes at 37 °C. The enzyme was removed and replaced with 2 mL of low ovomucoid solution (low-ovo), consisting of a 1.5% BSA (Sigma), 1.5% trypsin inhibitor (Worthington) and 0.07% DNAse D-PBS (Gibco) solution. A rabbit anti-rat macrophage antibody (80 μL, Cedarlane) was then added to the remaining 6 mL of low-ovo solution and mixed thoroughly. The low-ovo solution was aspirated from the retinas and a fresh 1 mL of the remaining low-ovo/anti-rat macrophage solution was added. The retinas were then gently triturated four times with a 1 mL pipette before adding a further 1 mL of low-ovo solution, with the top 1 mL then collected into a fresh falcon tube after the cells had settled. This was repeated until all of the 6 mL of low-ovo solution was used.

The 6 mL of dissociated retinal cells was left to incubate at room temperature for 10 minutes to allow binding of the anti-rat macrophage antibody before being centrifuged for 12 min at 1,000 RPM. The supernatant was removed, and the cells were re-suspended in 6 mL of a high-ovomucoid D-PBS solution (high-ovo) containing 3% BSA and 3% trypsin inhibitor, before being returned to the centrifuge for a further 12 minutes. The high-ovo solution was removed and replaced with panning buffer made from D-PBS containing 0.01% BSA and insulin (5 μg/mL, Sigma). The panning buffer cell suspension was then passed through a 20-micron filter (pluriSelect). The filtered cells were poured onto a 15 cm negative panning dish pre-coated with goat anti-rabbit IgG (H + L) (Jackson Immunoresearch) and left at room temperature for 15 minutes, before transferring the solution of non-adherent cells onto a second pre-coated negative panning plate for a further 45 minutes, shaking every 15 minutes. The macrophage depleted retina

cell solution was then transferred onto 10 cm positive panning plate, pre-coated with goat anti-mouse IgG (H + L) (Jackson Immunoresearch) and an anti-Thy1.1 antibody (clone OX-7, Sigma) and left for another 45 minutes, shaking every 15 minutes.

The positive plate was aspirated and gently washed several times with D-PBS so only adherent cells remained. The plate was rinsed with calcium-free EBBS (Sigma) before incubating in 0.05% trypsin-EDTA for approximately 4 minutes at 37 °C until adherent cells shook off. The solution was collected into a falcon tube with D-PBS containing 30% fetal bovine serum (Gibco) and centrifuged at 1,000 RPM for 12 minutes. The supernatant was aspirated, and the retinal ganglion cells were resuspended in 1 mL 1% NBA and counted. They were then plated at a density of 50,000-100,000 cells per coverslip in RGC growth media consisting of 1% NBA with added forskolin (4.2 μL, Tocris), BDNF (50 ng/mL) and CNTF (10 ng/mL), onto coverslips in 24 well plates pre-coated with poly-d-lysine and laminin, or else onto coverslips pre-seeded with a bed of mouse astrocytes. Cells were fed with fresh RGC growth media every four days.

2.3 Transfections and plasmids

Neurons and astrocytes were transfected in TMITS media (as described in *Chapter 2.1*) using Lipofectamine 2000 (2.33 μL/well, 1 μg/mL, Life Technologies), with a total plasmid concentration of 0.60 – 0.65 μg/mL. The Lipofectamine 2000/plasmid mix was incubated at room temperature for 20 minutes, before adding TMITS to give a total volume of 300 μL/well. Media from the wells to be transfected was removed (and saved aside if from neurons) before adding 300 μL of plasmid solution to each well. For astrocyte transfections, cells were transfected 48 hours after their plate down onto coverslips (before neuronal co-culture) and incubated with the plasmid mixture for 45 minutes at 37 °C, before being aspirated and fed with 1 mL DMEM media. For neuronal transfections, cells were transfected with green fluorescent protein (GFP) on DIV4 and left in the plasmid mixture for 2 hours at 37 °C before aspiration. They were then fed with saved media and topped up with an additional 1 mL of fresh 1% NBA (+AraC) media. The following plasmids were used in this thesis: CBF1_VP16 (RBP-J) was a gift from Tasuku Honjo (Kato et al., 1997), K_{IR}2.1 was a gift from Matthew Nolan.

2.4 Electrophysiological recordings

2.4.1 Solutions

Standard electrophysiological recordings were performed in an external solution of artificial cerebrospinal fluid (aCSF) containing (in mM, all Sigma): 150 NaCl, 2.8 KCl, 10 Na-HEPES, 2 $CaCl_2$, 1 $MgCl_2$ & 10 glucose, and pH adjusted to 7.3 with NaOH. For RGC recordings, the aCSF contained (in mM): 150 NaCl, 2.8 KCl, 10 Na-HEPES, 2.5 $CaCl_2$, 2 $MgCl_2$ & 10 glucose, pH adjusted to 7.3 with NaOH.

Internal recording solutions for astrocytes consisted of (in mM, all Sigma): 130 KCl, 4 glucose, 10 Na-HEPES, 0.1 EGTA, 0.025 $CaCl_2$, 20 sucrose, pH set to 7.2 with KOH, and osmolarity set to ~290 mOsm by addition of sucrose. For neuronal current-clamp and intrinsic property recordings a K-gluconate internal solution was used consisting of (in mM): 130 K-gluconate, 4 glucose, 10 Na-HEPES, 0.1 EGTA, 0.025 $CaCl_2$, 20 sucrose, pH adjusted to 7.3 with KOH. For neuronal and RGC voltage-clamp recordings of spontaneous activity and mEPSCs, a Cs-gluconate internal solution with 8 mM Cl^- was used, consisting of (in mM): 140 Cs-gluconate, 3 CsCl, 0.2 EGTA, 10 HEPES, 5 QX-314 chloride, 2 Mg-ATP, 2 Na-ATP, 0.3 Na_2GTP, 10 phosphocreatine, pH adjusted to ~7.4 with CsOH.

2.4.2 General recording set-up

All recordings were performed at room temperature (20-23 °C), using an Axopatch 200B amplifier (Molecular Devices), low pass filtered at either 5 kHz (for all astrocyte and neuronal intrinsic property/current-clamp recordings) or 2 kHz (for neuronal and RGC mEPSC recordings), and digitized at 5 kHz (spontaneous neuronal voltage-clamp recordings), 10 kHz (astrocyte and mEPSC recordings) or 50 kHz (intrinsic property current-clamp recordings) through a National Instruments BNC-2090 analogue-digital interface (National Instruments). All astrocyte, RGC and neuronal mEPSC and spontaneous activity experiments were recorded using WinEDR software (V3.7.5), and all neuronal intrinsic property/current-clamp experiments were recorded using WinWCP (V5.2.7; both Strathclyde Electrophysiology Software).

Perfusion of aCSF was achieved using a gravity fed set-up, at a rate of ~2 mL/min, with the inflow positioned ~2 mm away from the cell and the outflow located ~10 mm on the opposing side to

achieve efficient flow across the recorded cell. The perfusion system had six aCSF holding chambers, which were individually controlled using a six-channel valve controller (Warner Instrument Corporation) to rapidly apply drugs or change recording aCSF solutions.

Recording electrodes for whole-cell patch-clamp were pulled on a Model P-87 Flaming / Brown Micropipette Puller (Sutter Instruments), using thick-walled borosilicate glass of dimensions: AlisonA1.5 mm OD x 0.86 mm ID (Harvard Apparatus). Electrodes for astrocytic recordings had tip resistances of ~7-8 MΩ, and electrodes for neuronal recordings had resistances of ~4 MΩ.

Membrane potentials given in the results have not been corrected for the liquid junction potential (LJP) of the internal pipette solutions used. The LJP is approximately -4.6 mV for recordings using the KCl based internal solution (astrocyte recordings), and ~-16 mV for the gluconate-based solutions used (neuronal and RGC recordings). As such the true membrane potentials are more hyperpolarised than represented in the figures, for example the true neuronal RMP average in *Figure 4.3* will be approximately -63 to -67 mV.

2.4.3 Astrocyte recordings

Astrocytes were whole-cell voltage-clamped at -80 mV. Upon break-in, the amplifier was switched to current-clamp with I = 0 to take the resting membrane potential (RMP), before being returned to voltage-clamp, whereupon a current-voltage protocol (from -110 mV to 0 mV in 10 mV steps) was run to check cell identity and to determine the membrane resistance (R_M). The cell was then recorded in voltage-clamp at -80 mV whilst the perfusion of aCSF was run. EAAT currents were induced by rapidly applying aCSF containing L-Asp (200 μM, Sigma) for approximately 5 s, and then returning to aCSF. L-Asp application was repeated 3 times, and on the final application the L-Asp perfusion was instead switched to a solution of aCSF containing both L-Asp (200 μM) as well as the EAAT antagonist TFB-TBOA (20 μM, Tocris), to confirm current identity. For recording transfected astrocytes, cells positively expressing CBF1_VP16-GFP or Globin-GFP were visually identified by excitation with 467 nm light from a Colibri 7 LED system (Zeiss). Cells with RMP depolarised below -70 mV at break in, or calculated R_M > 100 MΩ, were discarded from analysis.

2.4.4 Neuronal recordings: intrinsic properties and excitability

Neurons were whole-cell voltage-clamped at -60 mV, and their RMP taken immediately upon break-in. From voltage-clamp at -60 mV a current-voltage relationship protocol was run (-80 to -50 mV, 5 mV steps) before current-clamping the neurons at -60 mV. A current injection stimulus protocol was run to determine the rheobase and to generate the frequency-input relationship. Current was injected for 0.5 s pulses, from -50 pA to +140 pA in 10 pA steps, with 5 seconds recovery at current-clamp I = -60 mV between each injection. The protocol was repeated to take an average result, with the access resistance checked between each recording.

For drug application experiments the above procedure was followed, before the RMP and current-voltage relationships were re-taken. Standard aCSF perfusion was then switched to the drug containing perfusion, and after 5 minutes of drug application the RMP and current-voltage relationships were repeated. The cell was then returned to current-clamp I = -60 mV and the current injection protocol repeated a further two times. The drugs used in this experiment were tertiapin Q (15 nM, abcam), ML 133 hydrochloride (4 μM, Tocris) and $BaCl_2$ (5 μM, Sigma). Cells whose access resistance changed >30% during recording were discarded.

2.4.5 Neuronal recordings: spontaneous activity and mEPSC

The RMP was taken immediately after break-in and neurons were then voltage-clamped at -60 mV. The spontaneous activity was first recorded with standard aCSF perfusing, with a test pulse injected every 2 minutes to monitor the access resistance. Recording times varied from 2-10 minutes depending on the activity level of the cell. For mEPSC recordings the standard aCSF was then switched for aCSF containing 300 nM tetrodotoxin (TTX) and 50 μM picrotoxin (PTX, Tocris), and mEPSCs were recorded for a further 2-10 minutes, checking the access every 2 minutes. If the access resistance was >30 MΩ or changed >20% during recording the cell was discarded.

2.5 Western blotting

Samples were collected in RIPA buffer and stored at -20 °C. On the day of use, the samples were defrosted on ice and a colorimetric BCA assay (Pierce) was run to determine the protein concentration of the samples (measured with a FLUOstar Omega microplate reader). Aliquots of

the samples were then taken and diluted in dH_2O to a concentration of 32.5 μg/65 μL. Reducing agent (10 μL, NuPAGE) and sample buffer (25 μL, NuPAGE) were added to 65 μL of the diluted sample (final protein concentration 10 μg/20 μL). The samples were then vortexed, spun down and boiled for 10 minutes.

Samples were vortexed again before being loaded into a 4-12% Bis-Tris Protein Gel (NuPage), at a volume of 20 μL per well, with ladder loaded into the outside lanes (10 μL, SeeBlue). Electrophoresis was run using MOPS buffer at 120 V. Once the gel had run, proteins were transferred onto a methanol activated PVDF membrane (Millipore) using transfer buffer (96 mM glycine, 12 mM Tris and 20% Methanol) at 80 V for 1 hour. Following transfer, the membrane was blocked in TBS-T solution (20 mM Tris, 137 mM NaCl and 0.1% Tween-20, pH 7.6) containing 5% milk for 1 hour at room temperature. The membrane was then cut in half along the ~35 kDa level, and each half incubated in its respective primary antibody overnight at 4 °C. Primary antibodies used were: Anti-Calmodulin (1 μg/mL; Merck 05-173) and Anti-GIRK1 (1:5,000; ab129182).

The following day the membranes were washed 3 times in TBS-T, and then incubated for 2 hours at room temperature in 10 mL of TBS-T +5% milk block with the appropriate HRP-linked secondary antibodies. Secondaries used were anti-rabbit IgG HRP-linked antibody (1:1000, Cell Signalling Technologies 7074) and polyclonal goat anti-mouse HRP (1:500, Dako P0447). Following secondary application, the membrane was washed 3 times in TBS-T, exposed to enhanced chemiluminescent reagents (LumiGlo) for 1 minute, and developed manually on Carestream BIOMAX light film. Blots were digitally scanned and densitometric analysis was performed using ImageJ.

2.6 Conditioned media experiments

To generate astrocytic conditioned media (ACM) for application to neuronal cultures, on astrocyte DIV11 the flasks were split and plated down in 1% NBA instead of DMEM. On neuronal DIV0 a proportion of this astrocytic media was pipetted from the astrocytes' wells, collected in a falcon tube, and dosed with AraC. After aspirating Opti-MEM from the neuronal samples, this treated ACM was used to feed the neurons instead of standard 1 % NBA. Astrocyte plates were topped up

with fresh 1% NBA and maintained alongside neurons. For neuronal DIV4 feeding, media was again collected from the astrocyte plate, treated with AraC, and fed to the neurons.

To generate ACM for mass spectrometry analysis, on astrocyte DIV7, 1x 75 cm^2 flask was split and plated into 1x 225 cm^2 flask, and on DIV11 this flask was split again (in DMEM) into 2x 225 cm^2 flasks. After 48 hours the DMEM was removed, the flasks washed 3x with D-PBS and 1x with their respective collection medias, and then 20 mL of their collection media was added. The two different sample collection medias used were Neurobasal-A minus phenol red (Gibco) with added glutamine (1 mM) and Anti-Anti (1%), and phenol-red free DMEM (Gibco) with added Anti-Anti (1%). Three days later the sample was collected from each flask into chilled 50 mL falcon tubes containing protease inhibitor (cOmplete). All following steps were carried out on ice. Samples were centrifuged at 2,000 g for 20 minutes at 4 °C, and then the supernatant was passed through a sterile 0.22 μm filter (Fisher) into a chilled falcon. Fifteen mL of the sample was then added to the reservoir chamber of a pre-chilled 3K centrifugal tube (Amicon), before being centrifuged at 4,000 g for 30 minutes at 4 °C. The filtrate was discarded, and the reservoir topped up to 15 mL with chilled dH_20 before returning to the centrifuge for a further 45 minutes. The concentrated samples were recovered into chilled Eppendorf tubes, a BCA assay run to determine protein concentration, before being stored at -80 °C.

To generate neuronal conditioned media, 20 mL of rat suspension was plated into a 75 cm^2 flask and fed with 1% NBA + AraC on DIV0 and DIV4. On DIV5 the flask was washed 3x with D-PBS, once with the sample conditioning media (phenol red-free NBA), and then 10 mL of the conditioning media was applied. On DIV7 the media was collected and concentrated as per the ACM protocol.

2.7 Immunohistochemistry and confocal imaging

On DIV14 samples were fixed in 1% formaldehyde for 20 minutes, washed 3 times with PBS, permeabilised with 0.5% NP40 (Life Technologies) for 5 minutes and then blocked in 1% BSA (Sigma) for 15 minutes. Antibodies were applied in 1% block solution, with 300 μL of solution applied to each well. The following antibodies were included in the solution: Synapsin 1 polyclonal rabbit antibody (1:1,000, SySy 106 103), Homer 1 monoclonal mouse antibody (1:200,

SySy 160 011) and fluorescein isothiocyanate (FITC)-conjugated GFP (1:500, abcam, ab6662). The plate was wrapped in foil and left to rotate at 4 °C overnight. The following morning the primary antibody solution was collected and saved, the wells washed three times with PBS, and then 300 μL of 1% block solution containing secondary antibodies was applied to each well. The secondaries used were: anti-mouse Cy3 (1:500, Jackson ImmunoReseach, 115-165-044) and anti-rabbit Alexa Flour 647 (1:1,000, abcam, ab150083). The plates were left rotating in secondary antibodies for two hours at room temperature. The coverslips were then washed five times in PBS and mounted on glass microscopy slides using Vectashield mounting media (with DAPI). The slides were covered and sealed from light and left at room temperature for two hours for the mounting medium to set, then stored at 4 °C in a slide box.

The samples were later imaged for synaptic markers on a Nikon A1R FLIM confocal microscope. GFP positive cells were selected under 10x magnification, before switching to an oil-submerged 60x lens to record processes of interest. For each recorded cell, 3 dendrites were selected and imaged at approximately 100 μm out from the cell body, which was typically on a secondary or tertiary branch. Around five cells were imaged per coverslip, across three independent culture batches. Images were analysed offline in Fiji (ImageJ), counting co-localised pre- and post- synaptic markers that occurred along the GFP positive dendrite region imaged. Co-localised puncta appeared as purple-yellow marks, where the pre-synaptic (blue) post-synaptic (red) and dendrite (green) markers overlapped. The number of synapses counted for each dendrite was divided by the length of the region and multiplied to give the number of synapses per 10 μm length. The average number of synaptic puncta/10 μm across the three dendrites per cell was taken to get the cell average.

2.8 RNA-sequencing

The samples that generated the RNA-sequencing data set of neuronal gene expression changes due to astrocytes were prepared and sent for sequencing by Dr Jing Qiu, University of Edinburgh. Briefly, pure rat neuronal cultures, or cultures of rat neurons on mouse astrocytes, were prepared as described in *Chapter 2.1*. Rat neuron and rat neuron-mouse astrocyte samples were collected on DIV8, with rat neuron cultures also collected at DIV14. The RNA was extracted from the samples, and converted into complementary strand DNA (cDNA), and sequenced by Edinburgh Genomics at a read depth of 50 million per species. The bioinformatic analysis on the returned sequencing was

conducted by Dr Owen Dando, University of Edinburgh. The reads were sorted by species, into rat or mouse, using the method as described in Hasel et al., 2017 and Qiu et al., 2018, with rat reads signifying neuronal expression, and mouse astrocytic.

The samples used to generate the sequencing data set of neuronal gene expression regulated by astrocyte conditioned media was prepared for RNA-sequencing by Dr Philip Hasel, New York University (formerly, University of Edinburgh). The prepared samples were sequenced by the Edinburgh Genomics Core Facility, and the bioinformatic analysis of the results was conducted by Dr Owen Dando and Dr Xin He, University of Edinburgh.

2.9 Mass spectrometry

ACM and NCM samples sent for secretomic profiling were sent to the MRC Institute of Genetics and Molecular Medicine (IGMM) Mass Spectrometry core facility at the University of Edinburgh, for untargeted proteomic analysis.

Briefly, at the core facility samples were adjusted to 6M guanidine HCl/tris (pH 8.5). Reduction/alkylation was acheived by addition to 5 mM TCEP and 10 mM CAA, before heating samples to 95 °C for 5 minutes. After cooling to room temperature, LysC (0.2 μg) was added and samples were diluted to 3M guanidine and incubated overnight at 37 °C. Trypsin (0.3 μg) was then added and samples diluted to 1M guanidine before a final incubation of 4 hours at 37 °C. Resulting peptides were cleaned on C18 Stage Tips. Peptides were separated for 40 minutes on a gradient 4-30 % acetonitrile (0.5% acetic acid throughout) using a Thermo Ultimate 3000-series RSLC Nano coupled with an IonOpticks Aurora C18 nano packed emitter in a Proxeon nano source fitted with Sonation column oven at 50 °C.

Ionised peptides were analysed on a Thermo Fusion Lumos in data-dependent mode and identified/quantified using MaxQuant version 1.6.7.0 with release 2019_07 of Uniprot Mouse and Human proteomes. Statistical analysis was performed using Wasim Aftab's implementation of Kammers et al., (2015) Linear Models for Microarray Data (LIMMA) pipeline for two group comparison in a proteomic experiment (Aftab, 2018).

Six concentrated ACM samples (3x replicates of NBA based ACM, 3x replicates of DMEM based ACM) and three samples of concentrated NCM (NBA based) were screened. There was little difference between NBA and DMEM conditions for ACM, so the results for the 6 samples were averaged together to give the relative amount of each detected protein in ACM, and the 3 neuronal samples were averaged to give the NCM protein levels.

2.10 Data analysis and statistics

Electrophysiology analysis was run using Stimfit (v0.15.4) software. In-built analysis functions were used to extract the data from IV relationships and FI protocols, which was then exported to Excel for further analysis.

For spontaneous activity and mEPSC analysis, a custom noise filtering script was written and implemented in Stimfit. Basically, a Fast Fourier Transform is run over the recording to detect constant sources of noise. The noise waves are then subtracted from the recording, to improve the clarity of the trace. For spontaneous activity, large events that have over 200 pC charge passing through them are first detected, removed and recorded as events, providing details such as the charge and amplitude of each event. A mini analysis script is then run to detect remaining small spontaneous events, extracting them and recording details including their amplitude, time constant and charge. The total charge, from both large and small events, for the recording is than calculated, as well as the average amplitude from all events. For mEPSC recordings, the noise protocol and mini analysis script is run. The results are then exported to Excel for further analysis.

All results are given as mean ± standard error of mean (SEM), unless otherwise stated. Basic statistical analysis, such as paired and unpaired Student's t-tests, were run in Excel. For most analysis a linear mixed effects (LME) analysis of variance (ANOVA) model was run using R statistical software. This analysis was set up to take variation between the different cultures into consideration. Each data point was coded for the culture batch it was taken from, and the variation in results due to week by week culture variation was then incorporated as a random variable into the statistical analysis. This is particularly important for this work, as many of the variables being measured, such as activity and synapse number, are dependent on culture density and health, with culture to culture variations having large effects on results between weeks (often larger than the effect measured

between tested conditions). Whereas a standard ANOVA is unable to take the random variation between these culture "clusters" into account, a mixed effect model can, making it the more powerful and appropriate model for the datasets presented in this thesis.

2.11 Ethics

Animal breeding, maintenance and procedures were performed in accordance with UK Animals (Scientific Procedures) Act 1986, under project license number P1351480E.

Chapter 3

Neuron to astrocyte signalling: neurons control functional expression of astrocytic glutamate transporters EAAT1 and EAAT2

Chapter 3 – Neuron to astrocyte signalling: Neurons control functional expression of astrocytic glutamate transporters EAAT1 and EAAT2

3.1 Introduction

Several instances of neuronal control of astrocytic form and function have been previously described. This includes the dramatic morphological changes that cultured astrocytes undergo in the presence of neurons (Hatten, 1985). Functionally there has also been an observed increase in glutamate transport in astrocytes grown in the presence of a neuronal feeder layer (Swanson et al., 1997, Gegelashvili et al., 1997, Drejer et al., 1983).

Although these early studies hint at the communication between these two major CNS cell types, a thorough exploration into the full extent of neuronal control of astrocyte function had yet to be undertaken. To explore this non-cell-autonomous signalling from neurons to astrocytes our laboratory used a mixed species culture approach to elucidate the impact of neurons on astrocytic gene expression (Hasel et al., 2017). We found that neurons both up and downregulated 1,000's of astrocytic genes (see *Figure 1.1)*.

Prominently among these upregulated astrocytic genes were genes associated with neurotransmitter uptake and metabolism. In line with previous studies showing an increase of astrocytic glutamate transport in the presence of neurons we found that both astrocytic glutamate transporter genes, *Slc1a2* (EAAT2) and *Slc1a3* (EAAT1), were significantly upregulated by neurons. Other genes associated with the glutamate and GABA pathways, such as glutamine synthetase (*Glul*), glutamate dehydrogenase (*Gad1*) as well as the GABA transporters GAT1 & 3 (*Slc6a1 & Slc6a11*), were also upregulated in the presence of neurons (Hasel et al., 2017).

Given the functional importance of astrocytic glutamate uptake in CNS health and disease, I first sought to investigate whether this change in gene expression was associated with a functional increase in glutamate uptake. Secondly, I sought to establish the signalling pathway from neurons

that induced this increase in astrocytic EAAT expression, and thirdly whether this signalling was required throughout development to maintain functional glutamate clearance.

I show that under our mixed species co-culture approach rat neurons can induce the dramatic morphological changes in mouse astrocytes that has been previously observed in same-species systems. I then demonstrate that in the presence of neurons, astrocytes display an enhanced glutamate uptake capability, and that this increase in uptake is in part mediated by neuron-dependent Notch signalling. I additionally show that neuronal Notch signalling is required throughout development to maintain astrocytic glutamate transporter function.

NB: throughout this chapter I refer to mouse astrocytes cultured alone as **mono-culture (MC) astrocytes**, and mouse astrocytes that have had rat neurons seeded on-top as **co-culture (CC) astrocytes.**

Much of the data in this chapter is published in Hasel et al., 2017.

3.2 Neurons alter astrocyte morphology

From the first days of pure astrocyte cultures it was observed that astrocytes grown alone appear as flat "fried-egg" or "pancake" shaped cells (Hatten, 1985). This is in stark contrast to their *in vivo* morphology, where they display a highly branched and complex star-like structure, from which they derive their name. However, growing these *in vitro* astrocytes in the presence of neurons transforms the astrocytes, pushing them towards the complex stellar structures that are observed in the brain. We previously confirmed that our rat neurons retained the ability to induce these same morphological changes to our mouse astrocytes (Hasel et al., 2017).

Examples of the *in vitro* MC and CC astrocytes that are used for the experiments throughout this chapter are shown in *Figure 3.1*. The above noted structural changes can be clearly seen.

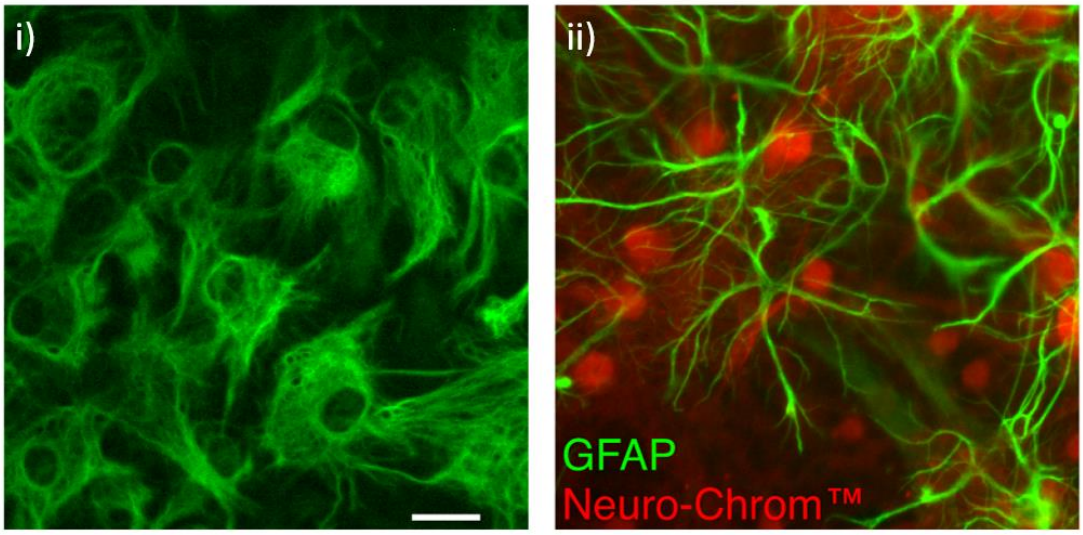


Figure 3.1: Neurons alter astrocyte morphology, increasing astrocytic complexity

Mouse astrocytes in the absence and presence of rat neurons. i) MC astrocytes display a flat "pancake" morphology compared to ii) CC astrocytes grown in the presence of rat neurons which display a more complex branched morphology that is associated with astrocytes *in vivo*. Scale: 20 µm. Astrocytes are stained for GFAP (green channel), and neurons with Neurochrom™ (red channel). Image taken from Hasel et al., 2017.

3.3 Neurons do not alter the basic membrane properties of astrocytes

Given the dramatic effect of neurons on astrocytic gene expression, I first investigated whether this resulted in a change to the astrocytes' basic membrane properties.

It is believed that astrocytes' membrane properties are set by $K_{IR}4.1$, which is one of the highest expressed genes and proteins in astrocytes (Butt and Kalsi, 2006, Neusch et al., 2006). This channel acts as a leak channel, allowing the ready passage of K^+ through the cell's membrane (Hibino et al., 2010). Classically, *in vivo* astrocytes display hyperpolarised resting membrane potentials (RMP) close to the K^+ reversal potential of -80 mV, a slightly inward rectifying current-voltage (IV) curve, as well as low membrane resistance (R_M), in line with their properties being set by $K_{IR}4.1$, in addition to the absence of depolarisation induced currents.

In our RNA-seq data we observed no significant change in expression of the gene for $K_{IR}4.1$ (*Kcnj10*) in the presence of neurons, although it was one of the most highly expressed astrocytic genes. Given this, if $K_{IR}4.1$ was primarily responsible for setting the astrocytic membrane properties I predicted to see little difference between MC and CC astrocyte membrane properties.

To investigate the effect of neurons on astrocyte membrane properties I used whole-cell patch-clamp. I saw little difference in the IV relationship between MC and CC astrocytes (*Fig. 3.2 i)* and no difference between conditions in either the RMP or R_M (*Fig. 3.1 ii & iii*). As such, despite their large influence on astrocyte morphology and gene expression, neurons do not control the basic membrane properties of astrocytes.

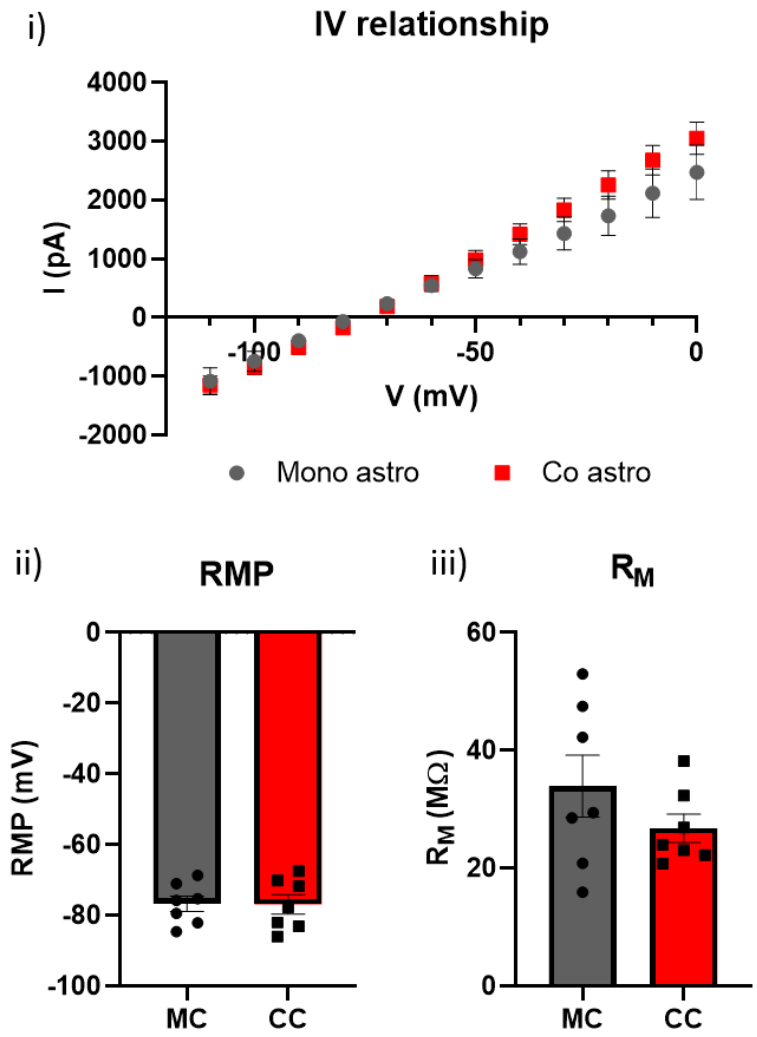


Figure 3.2: Neurons do not significantly alter the intrinsic properties of astrocytes

i) IV relationship of MC and CC astrocytes recorded at DIV8-10 (from neuronal plate down). ii) There was no significant difference between the RMP of MC and CC astrocytes (p = 0.58 unpaired Student's t-test, n = 7, 7 for MC and CC, respectively). iii) Likewise, there was no difference in the R_M (p = 0.28 unpaired Student's t-test, n = 7, 7 for MC and CC, respectively).

3.4 Neurons increase EAAT currents in astrocytes, which is prevented by inhibition of the Notch signalling pathway

To investigate whether there was an increase in astrocytic glutamate transporter function in CC astrocytes accompanying the increase at the mRNA level I used whole-cell voltage-clamp recordings. As the astrocytic glutamate transporters are electrogenic, with a net 2+ movement of charge per transport cycle, their activity can be measured as an inward movement of charge upon transporter activation (Zerangue and Kavanaugh, 1996).

To activate the transporters, I applied the EAAT substrate L-Aspartate (L-Asp) in the presence of the NMDA receptor antagonist AP-5 to minimise any channel activation. At the end of each recording I applied the high affinity EAAT antagonist TFB-TBOA (20 μM) to confirm L-Asp induced current identity.

L-Asp application to DIV8 MC astrocytes resulted in a small inward current (mean = 3.6 ± 1.0 pA, n = 16; *Fig. 3.3 i)*. This current was significantly greater when L-Asp was applied to DIV8 CC astrocytes (mean = 25.1 ± 3.4 pA, n = 19; *Fig. 3.3 ii & iv)*, showing that the increase in mRNA expression was associated with an increase in functional uptake.

Next, I wanted to find the signalling pathway behind this increase in EAAT expression. Work in the lab had shown that a prominent signalling pathway from neurons to astrocytes was the contact dependent Notch signalling pathway (Hasel et al., 2017; see *Fig. 1.5*). Additionally, we observed that inhibiting Notch signalling repressed not only the known downstream Notch genes *Hes5* and *Hey2*, but also *Slc1a2* (appendix *A1 iii*).

Although several pathways have been previously proposed for inducing astrocytic EAAT expression, I investigated whether the increase in Notch signalling observed in CC astrocytes also had a role in regulating functional EAAT activity in astrocytes. I inhibited Notch signalling in astrocytes by growing CC astrocytes in the presence of the γ-secretase inhibitor DAPT and then recorded their L-Asp induced currents. The induced EAAT currents in DIV8 CC astrocytes where Notch had been inhibited were significantly smaller compared to control CC astrocytes (mean = 6.9 ± 1.0 pA, n = 13; *Fig. 3.3 iii & iv*).

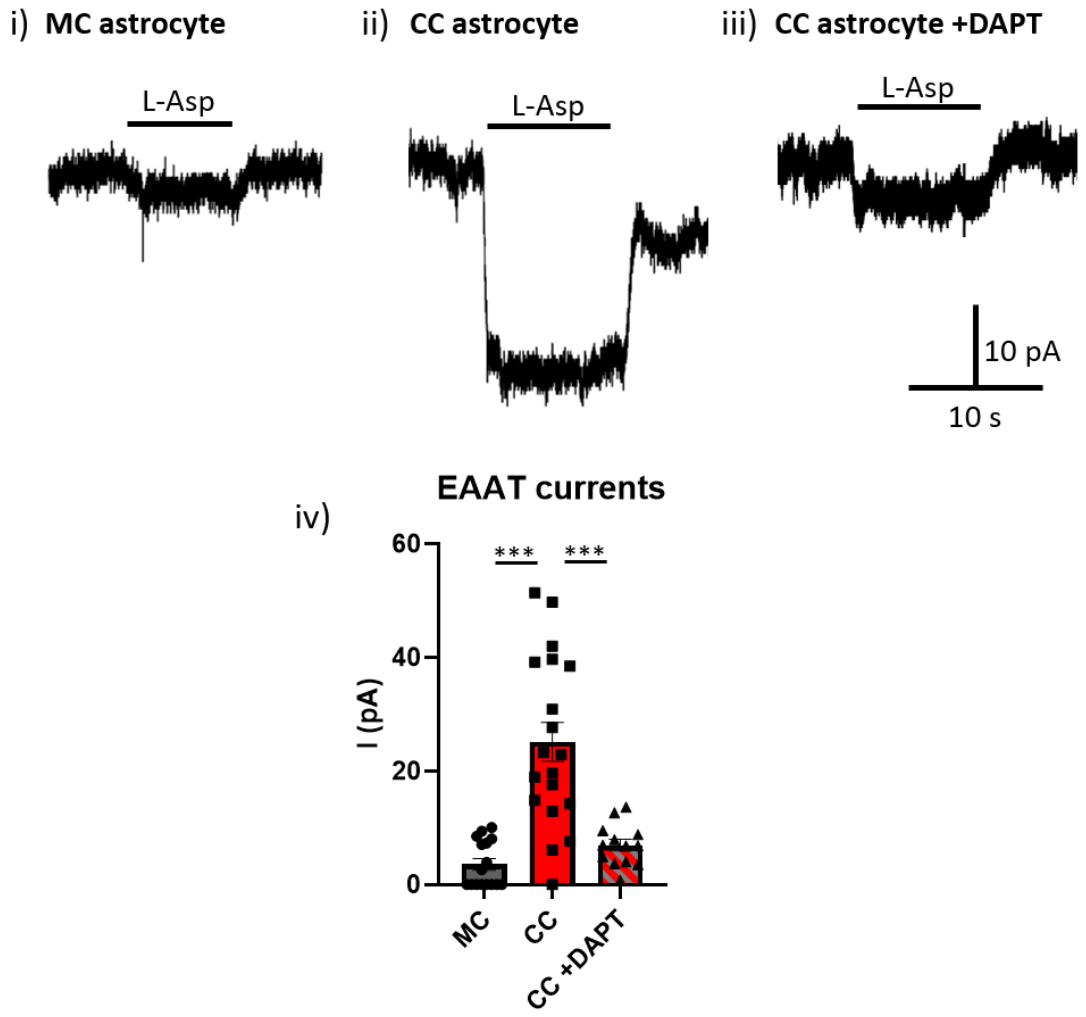


Figure 3.3: Neurons increase astrocytic EAAT currents, which is prevented by inhibition of Notch signalling

Astrocytic EAAT currents in DIV8-10 cultures (from neuronal plate-down) were induced by application of the EAAT substrate L-Asp (200 µM). i) Example trace of an L-Asp induced current in MC astrocytes, ii) CC astrocytes, and iii) CC astrocytes treated with the Notch signalling pathway inhibitor DAPT (10 µM) for 1 week. iv) Currents in CC astrocytes were significantly larger than currents in MC astrocytes ($p < 0.0001$, LME ANOVA, df = 42). Inhibition of Notch in CC astrocytes resulted in a significant decrease in EAAT currents ($p < 0.0001$, LME ANOVA, df = 42). All cells were voltage-clamped at -80 mV and recorded in the presence of 100 µM AP-5. Cells were recorded from 3 independent culture batches.

3.5 Activating Notch signalling in mono-culture astrocytes is sufficient to boost EAAT function

To confirm whether Notch signalling is involved in the increase in EAAT function in CC astrocytes I transfected MC astrocytes with the constitutively active form (CBF1_VP16) of the Notch effector, CBF1. Using a CBF1-luciferase reporter our lab had shown that expression of this plasmid in MC astrocytes was an effective way to boost Notch signalling activity in the absence of neurons (appendix *A1 iv*).

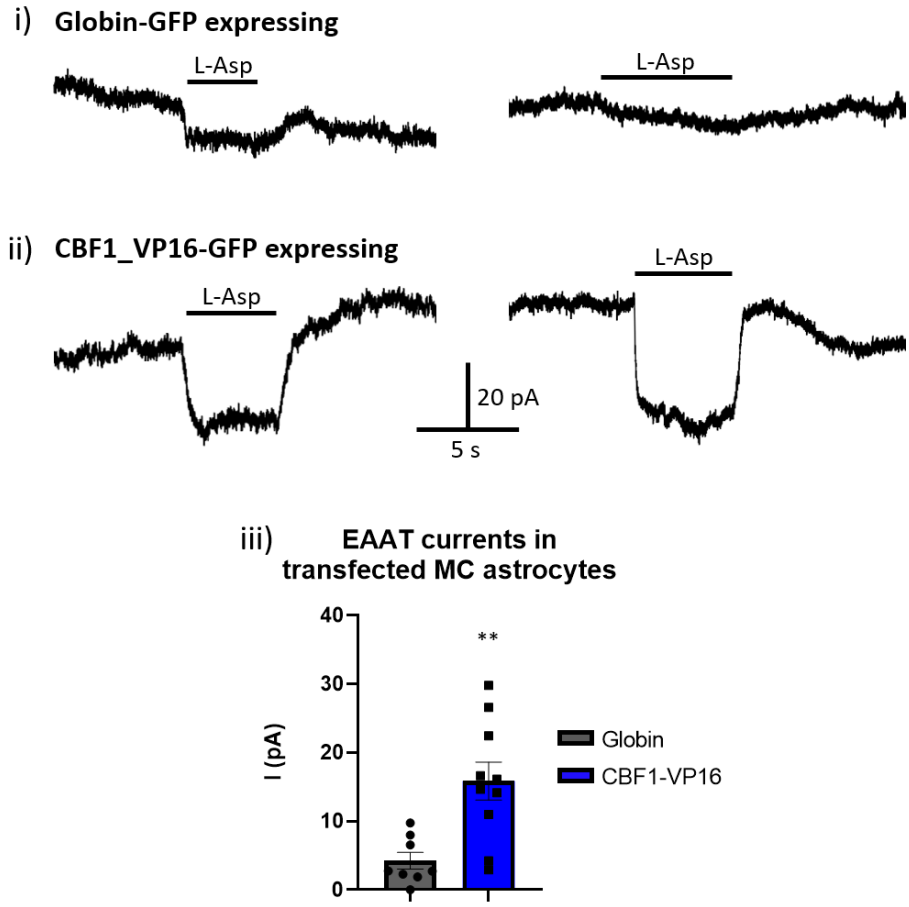


Figure 3.4: Activating Notch signalling in MC astrocytes is sufficient to boost EAAT activity

Mono-culture astrocytes were transfected with either the control plasmid globin-GFP or the constitutively active Notch effector CBF1_VP16-GFP 48 hrs after plate-down and recorded at DIV10. i) Example responses in control MC astrocytes and ii) MC astrocytes with active Notch signalling to L-Asp (200 μM). iii) MC astrocytes with activated Notch transcription displayed significantly larger responses to L-Asp (p = 0.003, Student's t-test, n = 8 & 10 cells for globin & CBF1_VP16, respectively, from 3 independent cultures). All cells were voltage-clamped at -80 mV and recorded in the presence of 100 μM AP-5.

I found that MC astrocytes expressing this active Notch effector had significantly larger responses to the EAAT substrate L-Asp than MC astrocytes expressing a globin control plasmid (15.8 ± 2.8 pA & 4.2 ± 1.2 pA, respectively; *Figure 3.4*). This demonstrates that activating the Notch transcription pathway in astrocytes is sufficient to boost EAAT function and provides an explanation for at least one signalling pathway from neurons to astrocytes that controls astrocytic EAAT expression and function.

3.6 Neuronal Notch signalling is needed throughout life to maintain EAAT expression

Up to now I've shown that signalling from neurons is required to induce astrocytic EAAT expression and function. This is an important function of astrocytes, with deterioration in astrocytic EAAT expression and function observed in neurodegenerative disease and aging (Masliah et al., 1996, Jacob et al., 2007, Trotti et al., 2001). One obvious question then is, is this signalling from neurons required throughout life to maintain EAAT expression, or is their expression set after their initial induction? If signalling is constantly required, then could faulty neuronal signalling be a potential cause of impaired astrocytic glutamate clearance?

To answer these questions, I grew MC and CC astrocytes out to a more mature timepoint, DIV22-25. I then blocked Notch signalling in some CC astrocytes from DIV0 with DAPT treatment, or, after recording currents at DIV13 to check the establishment of EAAT function, I applied DAPT treatment from DIV14. This allowed me to see whether blocking Notch signalling after EAAT currents were established a) did nothing, b) prevented any further changes to EAAT function, or c) reduced expression back down to MC levels.

By DIV13 CC astrocytic currents were shown to be well established and robust (mean = 209.3 ± 53.5 pA, n= 9 cells, 3 cultures; *Figure 3.5 I & vi*). In line with the results of *Chapter 3.4*, at DIV22 induced EAAT currents were significantly greater in CC astrocytes compared to MC astrocytes, albeit to a larger extent than seen at DIV8 (mean = 150.9 ± 34.0 vs 12.0 ± 7.0 pA, n = 9 & 6 cells, for CC & MC, respectively; *Figure 3.5 ii, iii & vi)*. Similarly, treatment of CC astrocytes with DAPT from DIV0 to prevent Notch activity resulted in a significant reduction in EAAT function (mean = 41.1 ± 13.8 pA, n = 7 cells; *Figure 3.5 iv & vi)*.

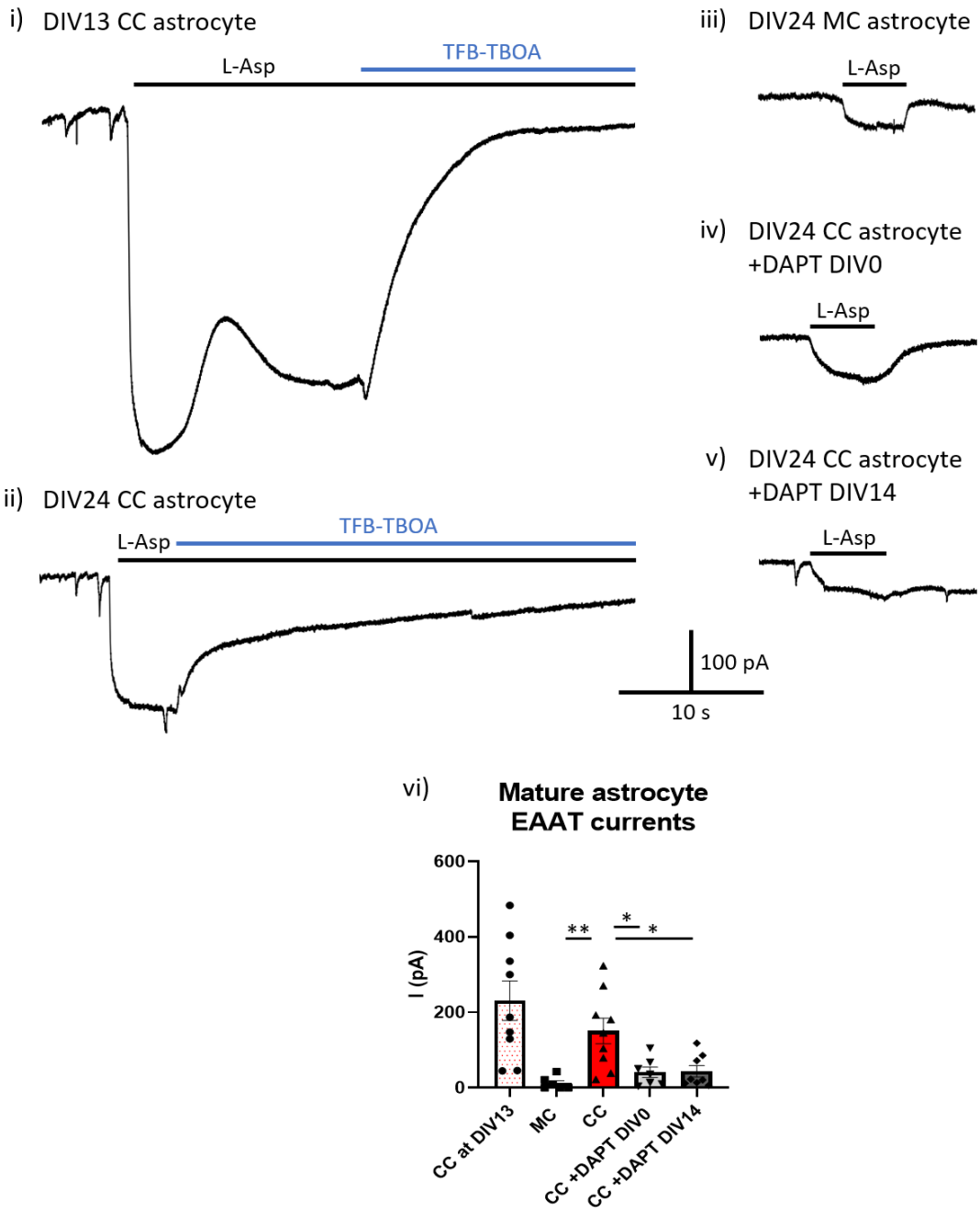


Figure 3.5: Notch signalling is needed to maintain astrocytic EAAT function

Example traces of EAAT mediated currents in response to 200 µM L-Asp in i) DIV13 CC astrocyte, ii) DIV24 CC astrocyte, iii) DIV24 MC astrocyte, iv) DIV24 astrocyte treated with DAPT from DIV0, and v) DIV24 astrocyte treated with DAPT from DIV14. Cells were voltage-clamped at -80 mV and all recordings were done in the presence of 100 µM AP-5. vi) At DIV22-25 there was a significantly larger EAAT mediated response in control CC astrocytes compared to MC astrocytes (p = 0.009, LME ANOVE, df = 29), as well as CC astrocytes treated with DAPT from either DIV0 or DIV14 (p = 0.028 & 0.027, for DIV0 and DIV14 respectively, LME ANOVA, df = 29). There was no difference in EAAT response in CC astrocytes treated with DAPT from DIV0, or from DIV14 after currents had been established. Recordings were taken from cells across at least 3 independent culture batches.

When CC astrocytes were only treated with DAPT from DIV14, after the establishment of EAAT function, their induced EAAT activity was significantly less than control CC astrocytes (mean = 43.9 ± 14.9 pA, n = 8 cells; *Fig. 3.5 v & vi)*. Moreover, the responses of the CC astrocytes with Notch inhibited after EAAT function had been established was indistinguishable from the responses of CC astrocytes that had Notch inhibited from DIV0 (41.1 ± 13.8 versus 43.9 ± 14.9 pA, $p = 0.95$, LME ANOVA, df = 29). Of note, the responses of both DAPT treated CC astrocytes appeared slightly greater than MC astrocytes to L-Asp, suggesting either incomplete Notch inhibition or the involvement of some other pathway in EAAT induction in astrocytes.

I've shown here that not only is Notch signalling from neurons required for the induction of EAAT transporter function, but it is also required throughout the astrocytes life in order to maintain EAAT functional activity.

3.7 Discussion

3.7.1 Summary of findings

I have shown in this chapter that cortical neurons control some important functional aspects of cortical astrocytes. Neurons do not alter the gene expression of the highly expressed $K_{IR}4.1$ channel, and correspondingly have little impact on the basic membrane properties of astrocytes. However, neurons significantly upregulate the expression of *Slc1a2* and *Slc1a3*, and I showed that this upregulation corresponded to a significant increase in the function of the astrocytic glutamate transporters, EAAT1 and EAAT2. I then demonstrated that neurons control this functional astrocytic EAAT expression via the contact-dependent Notch signalling pathway. Importantly, the work I presented in this chapter suggests that neuronal Notch signalling is continuously required in order to maintain astrocytic EAAT expression and function.

3.7.2 Neurons control astrocytic EAAT1 and EAAT2 through Notch signalling

Consistent with previous studies I have shown in this chapter that neurons increase the activity of astrocytic glutamate transporters. This finding was predicted from the results of RNA-sequencing work conducted by our lab, which saw a dramatic increase in the gene expression for these transporters, *Slc1a2* and *Slc1a3* (see *Figure 1.1*). The results of this sequencing additionally showed that the expression for *Kcnj10* ($K_{IR}4.1$) in astrocytes was relatively unaffected by the addition of

neurons. As $K_{IR}4.1$ is believed to play a significant role in determining astrocytic membrane properties I predicted that neurons should have little effect on astrocytic properties, which I found to be the case. Given these two findings, I wondered if the results of this sequencing might also provide a clue as to the signalling pathway by which neurons control the astrocytic glutamate transporters. Although several factors have been found to modulate the expression of astrocytic EAATs, the evidence is still inconclusive as to the physiological signalling pathway(s) that regulate(s) their expression. Given the fundamentally important role these transporters play in the nervous system, with their combined deletion being lethal and impairments in their function associated with disease, finding the pathway by which they are regulated remains of high imperative.

Within the group of astrocytic genes that were upregulated by neurons were *Hes5* and *Hey2*, known downstream products of the Notch signalling pathway. Investigating this further, I found astrocytes to highly express Notch receptors, whereas neurons expressed Notch ligands. These observations were suggestive of an astrocytic Notch signalling pathway being present in these cells that was activated by neurons, a finding confirmed by Dr Philip Hasel (appendix *A1 ii*). Therefore, I decided to investigate whether the Notch signalling pathway might be controlling the astrocytic glutamate transporters. I found that inhibiting Notch by preventing the cleavage of the NICD with the γ-secretase inhibitor DAPT strongly reduced the EAAT transporter function in the presence of neurons. This suggested that neuronal activation of Notch in astrocytes was responsible for inducing astrocytic EAAT function. If this pathway was responsible alone for astrocytic EAAT induction, then simply activating Notch in astrocytes should boost EAAT function independent of neuronal presence. To investigate this, I transfected astrocytes with the constitutively active form of the Notch effector – which results in increased Notch activity in transfected astrocytes (appendix *A1 iv*). Indeed, I found that simply turning on Notch in MC astrocytes, without neurons present, was sufficient to boost astrocytic EAAT function. This result also nicely confirmed the results of my earlier DAPT experiment, ruling out the possibility that the reduction in EAAT function was due to non-specific effects of γ-secretase treatment, rather than Notch inhibition *per se*. Overall, these results show that neurons increase astrocytic functional EAAT expression via the contact-dependent Notch signalling pathway.

My findings here suggest that the observations that neuronal Notch ligands induce the glial glutamate transporter EAAT1 in drosophila is a conserved signalling pathway in mammals (Stacey

et al., 2010). There is also the possibility that this induction of astrocytic EAAT transporters is not specific to neurons, as endothelial cells have also been shown to induce mouse astrocytic EAAT2 in culture (Lee et al., 2017). As well as astrocytes' close association with neurons, they also send their processes to blood vessels, so physiologically this presents the possibility that these endothelial cells also have a role in inducing astrocytic EAAT transporters in the brain. In relation to past work on astrocytic EAAT regulation, my results in this chapter give rise to a further interesting possibility. As discussed in *Chapter 1.5*, earlier studies had found that cAMP signalling was able to induce EAAT function in astrocytes in a similar manner to neuronal co-culture. However, how this cAMP induction related to the physiological neuronal signalling cascade that induced astrocytic EAAT had not been discovered. In light of a 2015 paper suggesting that cAMP increases NICD translocation and Notch pathway activity, it is certainly possible that the previously observed effects of cAMP on astrocytic EAAT induction may be due to enhancement of Notch activity (Angulo-Rojo et al., 2013). In future work it would be interesting to confirm whether the effects of cAMP on astrocytic EAAT function are in fact mediated by Notch signalling, which would solve the mystery of cAMP's physiological relationship to neuron induced astrocytic EAAT expression.

3.7.3 Notch signalling is needed to maintain astrocytic EAAT function

Importantly, my work has shown that Notch signalling is continually required to maintain astrocytic EAAT expression. Not only does Notch increase astrocytic EAAT function, its removal completely reverses this induction. The potential significance of this finding is in relation to the fact that astrocytic EAAT dysfunction and reductions in EAAT expression have been repeatedly associated with neurodegenerative diseases of all varieties (Van Den Bosch et al., 2006, Pál, 2018, Hoshi et al., 2018, Masliah et al., 1996, Li et al., 1997, Jacob et al., 2007). The link between impaired astrocytic glutamate clearance and neuronal loss due to excitotoxic insult has always been clear, and it seems obvious that such dysfunctions may in part contribute to the progression of neurodegeneration. My observation that removing Notch reduces astrocytic EAAT function presents the possibility that some neurodegenerative diseases may be the result of a snowball effect involving glutamate clearance, hence the typical appearance later in life followed by rapid decline. For example, something may trigger a reduction in neuron numbers or neuronal branching leading to a decrease in contact with astrocytes, reduced astrocytic Notch signalling and finally reduced EAAT expression. This reduced expression of EAATs may then lead to greater exposure of neurons

to extracellular glutamate, triggering apoptosis, resulting in greater loss of neurons and greater loss of contact with astrocytes, further reducing astrocytic EAAT expression. Similarly, small dysfunctions in the Notch signalling pathway, or small reductions in astrocytic EAAT function, may be what initially leads to neuronal death through prolonged elevated extracellular glutamate concentrations. This neuronal death then causes further reductions in Notch signalling and astrocytic EAAT function, exacerbating cell loss.

Support for this hypothesis comes from observations that impairments in γ-secretase function have long been associated with Alzheimer's disease. As well as γ-secretase's role in cleaving the NICD, it is also involved in cleavage of the amyloid precursor protein (APP); increased cleavage of APP into toxic Aβ fragments leads to the production of amyloid plaques, a characteristic feature of AD (Jurisch-Yaksi et al., 2013). As it turns out, mutations that cause familial early onset AD have predominantly been found to be in a component of the γ-secretase complex, namely presenilin-1 and -2 (Hutton and Hardy, 1997, Sassi et al., 2014). These mutations alter the activity of γ-secretase and cause an increase in production of toxic Aβ fragments. Therefore, an early human AD drug trial was with a broad spectrum γ-secretase inhibitor, with the belief that preventing toxic Aβ and amyloid production would improve disease (De Strooper, 2014). However, the opposite occurred: inhibiting γ-secretase *worsened* AD progression, with this worsening attributed to the incidental inhibition of Notch signalling (De Strooper, 2014, Henley et al., 2014). Significantly, presenilin mutations that give rise to early onset AD and increased pathogenic Aβ production are concurrently found to have a significantly reduced ability the cleave the NICD, suggesting that although mutant presenilin might enhance production of toxic Aβ it also results in loss of Notch activation (Moehlmann et al., 2002, Brai et al., 2016, Song et al., 1999, Chen et al., 2002). Further linking Notch-EAAT impairments to neurodegenerative diseases, a recent paper found significant reductions in NICD in hippocampal tissue of human ALS patients compared to controls (Gómez-Pinedo et al., 2019). This work complements our lab's findings of both reduced Notch pathway genes (*Hes5* and *Hey2*) and importantly reduced *Slc1a2* expression, in a mouse model of motor neuron disease, suggestive of reduced Notch signalling (Hasel et al., 2017). Therefore, as Notch signalling induces astrocyte EAAT functional expression, the reduced astrocytic *Slc1a2* and *Slc1a3* expression that is seen in AD and motor neuron disease may be due to reduced Notch activity, for example due to mutant γ-secretase activity. This reduction may then lead to the increased

excitability and glutamate dysregulation seen in patients, which may exacerbate neurodegeneration (Amatniek et al., 2006).

Several studies have now investigated the role of impaired Notch signalling in forebrain neurons in neurodegeneration, producing conflicting results. Early studies found that conditional knockout of either the nicastrin or presenilin components of γ-secretase specifically in forebrain excitatory neurons caused impairments in learning and memory followed by neurodegeneration (Tabuchi et al., 2009, Saura et al., 2004). However, two independent groups in 2012 who produced conditional knockouts of Notch receptors and the Notch effector CBF1 (RBPj) specifically in excitatory forebrain neurons found that inhibition of Notch signalling in these neurons had no impact on memory and did not lead to neurodegeneration (Zheng et al., 2012, Sato et al., 2012). On the surface these results suggest that the toxic effect of mutant γ-secretase is not due to reduced Notch signalling, but these findings do not rule out the possibility that impairments in astrocytic Notch signalling may be involved in the pathological development of disease. Interestingly, heterozygous $Notch1^{+/-}$ and $RBPj^{+/-}$ mice were found to have deficits in spatial memory and learning (homozygous knockouts being embryonic lethal), suggesting that reduced Notch signalling in cells other than forebrain neurons does lead to cognitive impairment (Costa et al., 2003). Given my results here demonstrating the requirement of Notch signalling for astrocytic EAAT function it would be interesting for future work to re-explore the involvement of Notch signalling in neurodegeneration, but this time investigating the role of impaired astrocytic Notch signalling, rather than neuronal.

Interestingly, as well as the reported reductions of astrocytic EAAT expression in neurodegenerative disease, it appears that there may be a natural decline in the expression of these transporters with age in humans. From a dataset produced by Barres and colleagues in 2016, where they reported the gene expression in astrocytes purified from healthy human CNS tissue (subjects ranging from 8 to 63 years old), the mean expression of the astrocytic glutamate transporters *SLC1A2* and *SLC1A3* was approximately 35% lower in the 6 samples from subjects >40 years old compared to the 6 samples from subjects <40 years old (see appendix *A10*; Zhang et al., 2016). Furthermore, the expression of Notch pathway related genes, including *HES5* and the γ-secretase components – in particular nicastrin, was approximately 30% lower in astrocytes from these older human samples (Zhang et al., 2016). These results suggest that during the course of human aging there is a reduction in Notch

signalling in astrocytes, resulting in a decrease in glutamate transporter expression. This further supports a link to impaired Notch signalling/astrocyte glutamate clearance and dementia, as the vast majority of cases of non-familial AD and dementia appear later in human life. Thus, exacerbation of these age-related reductions, for example due to lifestyle factors, may lead to the onset of dementia in sporadic cases.

It is an open question whether the decline in astrocytic Notch or glutamate clearance precedes neurodegeneration, or whether reductions in neurons and neurodegeneration lead to the reduction in Notch signalling and transporter expression. Either way, it would be intriguing to see whether *boosting* Notch signalling and EAAT expression in healthy older-aged humans (without familial AD) could prevent or reduce the later incidence of dementia.

3.7.4 Conclusions

Neurons are responsible for controlling the expression of the physiologically important glutamate transporters in astrocytes. They induce astrocytic EAAT1 and EAAT2 expression and function through the contact dependent Notch signalling pathway, with removal of this signalling causing a significant reduction in astrocytic EAAT function. Further work is required to assess whether the previously observed effects of cAMP on astrocytic EAAT function are due to an interaction with the Notch signalling pathway. Importantly, future investigations should focus on the potential involvement of reduced Notch signalling in astrocytes, and the downstream effects on astrocytic EAAT function, in the progression of neurodegeneration.

Chapter 4

Astrocytes alter neuronal gene expression and physiological properties

Chapter 4 – Astrocytes alter neuronal gene expression and physiological properties

4.1 Introduction

Historically astrocytes had clear functions ascribed to them, prominently their roles in glutamate clearance and K^+ buffering. Recently, the suggested roles of astrocytes in neuronal function have been greatly expanded, with astrocytes now believed to play important roles in directing neuronal development and more directly influencing neuronal function. One prominent theory suggests that astrocytes are required for excitatory synapse formation. This idea was first brought forward from early work on retinal ganglion cell (RGC) cultures, where it was observed that culturing RGCs in the presence of astrocytes lead to an increase in excitatory synapse formation (Pfrieger and Barres, 1997, Ullian et al., 2001). Since then a number of astrocyte secreted proteins have been proposed as excitatory synapse promoters, including thrombospondins, glypicans and SPARCL1, largely based on work with purified RGCs (Allen et al., 2012, Eroglu et al., 2009, Christopherson et al., 2005, Singh et al., 2016, Kucukdereli et al., 2011). The evidence for astrocyte dependent excitatory synapse formation in other brain regions, such as the cortex, is less clear. As well as their potential involvement in synapse development, astrocytes are believed to actively participate in neuronal activity by secreting neuro-modulatory chemicals, such as glutamate, in what has been termed the tri-partite synapse (Perea et al., 2009, Araque et al., 1999). Along these lines, there is evidence to suggest that astrocyte released factors are required for both LTP and LTD (Henneberger et al., 2010, Adamsky et al., 2018, Min and Nevian, 2012, Navarrete et al., 2019). However, despite the increased interest in how astrocytes communicate with neurons, and how this may alter neuronal function, no work has yet investigated how astrocytes control neuronal gene expression and the full extent of astrocytic-neuronal interactions.

Given the recent interest in astrocytes' roles in neuronal function and the lack of accompanying knowledge, I first sought to investigate how astrocytes may alter cortical neuronal gene expression. I employed a robust culture system that allows for healthy cortical neuronal growth and survival in the absence of astrocytes, in order to address the historical bias and limitations of using RGCs as a model for astrocyte-neuron interactions. I used a similar mixed-species approach with these cells as

in the previous chapter, this time culturing rat cortical neurons alone, or on a bed of mouse cortical astrocytes, combined with RNA-sequencing results from this system.

Under this set-up I found that cortical astrocytes influenced many cortical neuronal genes, but not always the genes expected. I used the lab's RNA-seq data to hypothesise how astrocytes might affect neuronal properties: of interest I found that astrocytes downregulated the expression of neuronal K^+ inward-rectifier channels (K_{IR}). As these channels are important for setting membrane properties, I hypothesised that this would lead to functional differences in neurons cultured with astrocytes leading to enhanced excitability. Indeed, I found that neurons cultured with astrocytes do have differences in their properties that led to an increase in their excitability, and that manipulating K_{IR}s is able to replicate these effects. Furthermore, these K^+ channels go on to be regulated in an activity-dependent way, but only in the presence of astrocytes. To investigate the mechanism behind this control of K_{IR} expression, I then used astrocyte conditioned media to show that an astrocyte secreted factor was responsible for these changes. Using unbiased proteomics, I then explored what factors in ACM may be responsible for the regulation of K_{IR} channels.

NB: Throughout *Chapter 4* I refer to rat neurons cultured alone as **mono-culture** (MC), and rat neurons cultured on mouse astrocytes as **co-culture** (CC). This is different to *Chapter 3*.

4.2 AraC treatment prevents astrocyte growth and proliferation

To generate astrocyte-free cortical neuronal mono-cultures the chemotherapy drug cytosine arabinoside (AraC) was used. AraC is able to incorporate itself into DNA during cell proliferation, and once in the cellular DNA induces cell death. As astrocytes proliferate, the addition of AraC to culture media is taken up by any currently proliferating astrocytes. At the point of plate down all astrocytes enter a phase of proliferation, and as such treatment with AraC effectively removes all astrocytes from the culture.

To generate a co-culture, rat cell suspension is plated on top of a bed of established mouse astrocytes. These astrocytes have had time to settle, and many are no longer undergoing proliferation, so treatment with AraC does not kill these established astrocytes off.

The effectiveness of this strategy is shown in *Figure 4.1*, with panels *i & iii* depicting rat suspension plated onto blank coverslips (mono-culture), and *ii & iv* depicting the same culture plated onto established mouse astrocytes (co-culture). Both conditions had been treated with AraC on DIV0 and DIV4, and at the time of collection (DIV15) it can be observed that the mono-culture is still free of astrocytes.

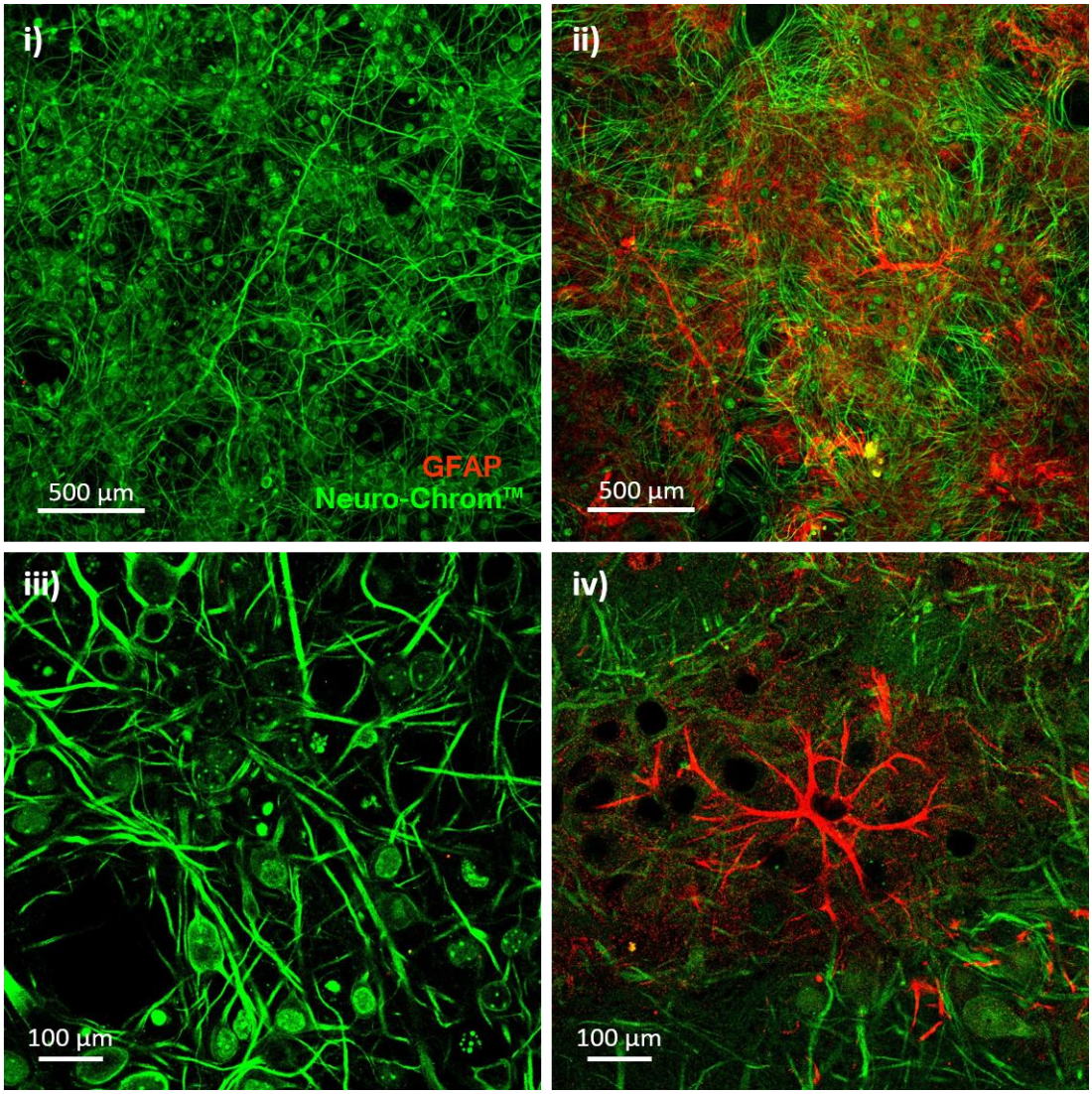


Figure 4.1: Rat neurons cultured in the absence and presence of mouse astrocytes, treated with AraC

Treatment with the cytotoxic agent AraC (4.8 µM) on rat DIV0 and DIV4 successfully prevents astrocyte proliferation. Panels i & iii show DIV15 rat cortical cells that were seeded onto coverslips. AraC treatment kills astrocytes in the rat suspension undergoing proliferation, preventing their survival, creating a neuron mono-culture. Panels ii & iv show DIV15 rat suspensions that were instead seeded onto an established bed of mouse astrocytes. AraC does not kill established mouse astrocytes no longer proliferating, creating a mixed species co-culture.

4.3 Investigating the effect of astrocytes on the neuronal transcriptome using mixed-species RNA-sequencing

To investigate how cortical astrocytes effect cortical neuronal gene expression, the lab employed a mixed species RNA-sequencing approach (as described in Hasel et al., 2017). Rat neurons were grown alone or on a bed of mouse astrocytes, samples collected and prepared, and then sent for sequencing. The resultant reads from the RNA-sequencing were sorted on species, using the difference in genome between mice and rats. The change in gene expression in rat reads with the addition of mouse astrocytes was then analysed, which represents the change in neuronal gene expression due to astrocytes.

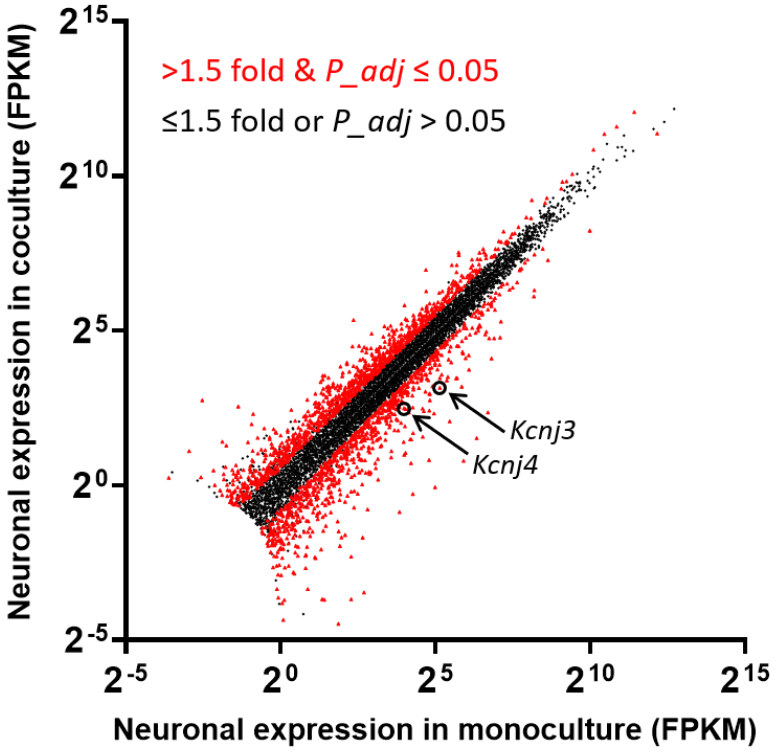


Figure 4.2: Change in neuronal gene expression due to astrocytes

Results from the mixed species RNA-seq of DIV8 rat neurons cultured alone or on top of mouse astrocytes. There was a total of 1,960 neuronal (rat) genes that were significantly up or downregulated >1.5 fold in the presence of astrocytes (out of a total of 13,178 rat genes). As a whole, the expression of the K_{IR} family of genes were downregulated by co-culture, with the most prominently expressed members in neurons, *Kcnj3* ($K_{IR}3.1$) and *Kcnj4* ($K_{IR}2.3$), showing the greatest degrees of downregulation. Data courtesy of Dr J. Qiu (experiment design and sample preparation) and Dr O. Dando (bioinformatic processing analyses).

The results of this analysis were sorted into genes with >1.5-fold change in expression with a significance of *P*_adjusted ≤ 0.05 in the presence of astrocytes, and those with either ≤1.5-fold change or *P*_adjusted > 0.05, as shown in *Figure 4.2*. Overall, 14.9% of cortical neuronal genes were significantly up- or downregulated by >1.5-fold by astrocytes.

Some of the most significantly upregulated genes were those for tubulins (appendix *A2*). Synaptic associated proteins, on the other hand, were largely unaffected by the presence of astrocytes (appendix *A3*). However, the K_{IR} family of genes as a whole were significantly downregulated in the presence of astrocytes (appendix *A4*). In particular the genes for $K_{IR}3.1$ and $K_{IR}2.3$, which were the most expressed K_{IR}s in neurons, were the most downregulated by astrocytes. This down regulation was not due to a hastening of maturation: gene expression of K_{IR}s was seen to *increase* from DIV8 to DIV15 in cortical neurons (appendix *A4*). Therefore, astrocytes impede K_{IR} expression in cortical neurons.

4.4 Neuronal membrane properties are altered in co-cultured neurons, consistent with a decrease in K_{IR} expression

Due to the change in cortical neuronal K_{IR} expression, I sought to establish if this led to physical changes in neuronal properties. First, I looked at the membrane and action potential properties of MC neurons compared to CC neurons. I found that the resting membrane potential (RMP) was significantly depolarised in CC cells compared to MC cells (*Fig 4.3 i*: mean = -52.1 ± 1.0 mV vs -47.9 ± 1.1 mV, n = 44 & 24, for MC and CC cells, respectively). I further found that the membrane resistance (R_M) was significantly higher in CC cells, consistent with a decrease in K_{IR} expression (*Fig 4.3 ii*: mean = 523.3 ± 37.1 vs 702.7 ± 60.6 MΩ, n = 44 & 24, for MC and CC respectively). I also saw that the amplitude of the first action potential to be induced was significantly higher in CC neurons (*Fig 4.3 iii*: mean = 69.1 ± 2.3 vs 81.8 ± 2.7 mV, for MC and CC respectively), although the mechanism behind this is not further explored in this work.

To check for any effects of AraC treatment on the CC cells, I had a third condition of CC cells grown without the addition of AraC. In these samples there is a mix of mouse and rat astrocytes along with the rat neurons. I found no difference between CC cells grown with or without AraC for either RMP, R_M or action potential amplitude.

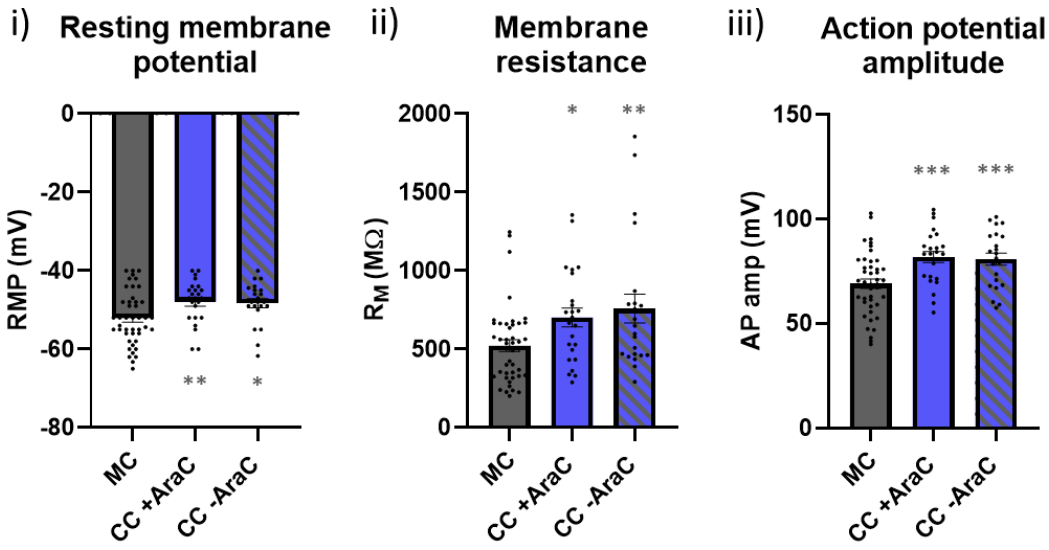


Figure 4.3: The presence of astrocytes alters the membrane properties of cortical neurons at DIV8

The membrane properties of rat cortical neurons were recorded at DIV8. Neurons were either grown in rat mono-culture (+AraC, n = 44 cells), rat neuron + mouse astrocyte co-culture (+AraC, n = 24 cells), or co-culture without AraC (-AraC, n = 22; rat neuron & rat astrocyte on mouse astrocyte) to control for any effects of AraC treatment. i) Both co-culture conditions caused a significant depolarisation in RMP compared to mono-culture ($p = 0.008$ and $p = 0.02$ for CC +AraC and CC -AraC, respectively, LME ANOVA, df = 79). ii) The membrane resistance in cortical neurons grown with astrocytes was also significantly increased ($p = 0.02$ and $p = 0.003$ for CC +AraC and CC -AraC, respectively), as well as the height of the first action potential ($p < 0.001$ and $p = 0.002$ for CC +AraC and CC -AraC, respectively). Cells were recorded from 9 independent culture batches.

4.5 Neuronal excitability is increased by the presence of astrocytes

Having observed my hypothesised changes in cortical neuronal membrane properties in the presence of astrocytes, I next investigated whether there was an effect of astrocytes on the excitability of these cells. To do this I examined the frequency-current (*FI*) relationship in MC and CC cells. This relationship is the measure of the number of action potentials elicited due to a given input current (with cells current-clamped at a specified holding potential, in this case -60 mV). The minimum current that is required to be injected to induce an action potential is known as the rheobase. An increase in a cell's excitability is then marked by a leftward shift in the *FI* relationship and a decrease in the rheobase: that is, less current is required to cross the cell's membrane to induce firing.

There was a leftward shift in the *FI* relationship between MC neurons and both CC neurons +AraC and CC neurons -AraC treatment, with significantly more action potentials fired in both CC conditions compared to MC for current injections ranging from 20 pA to 80 pA (*Fig 4.4 ii*). This was accompanied by a significant decrease in the rheobase for CC conditions (*Fig 4.4 iii*: mean current to fire an AP = 67.7 ± 4.1, 37.9 ± 4.9 and 32.3 ± 2.8 pA, n = 44, 24, 22, for MC, CC +AraC and CC -AraC, respectively). Overall, there is an increase in the excitability of cortical neurons grown in the presence of astrocytes, which is consistent with the observed changes in their membrane properties.

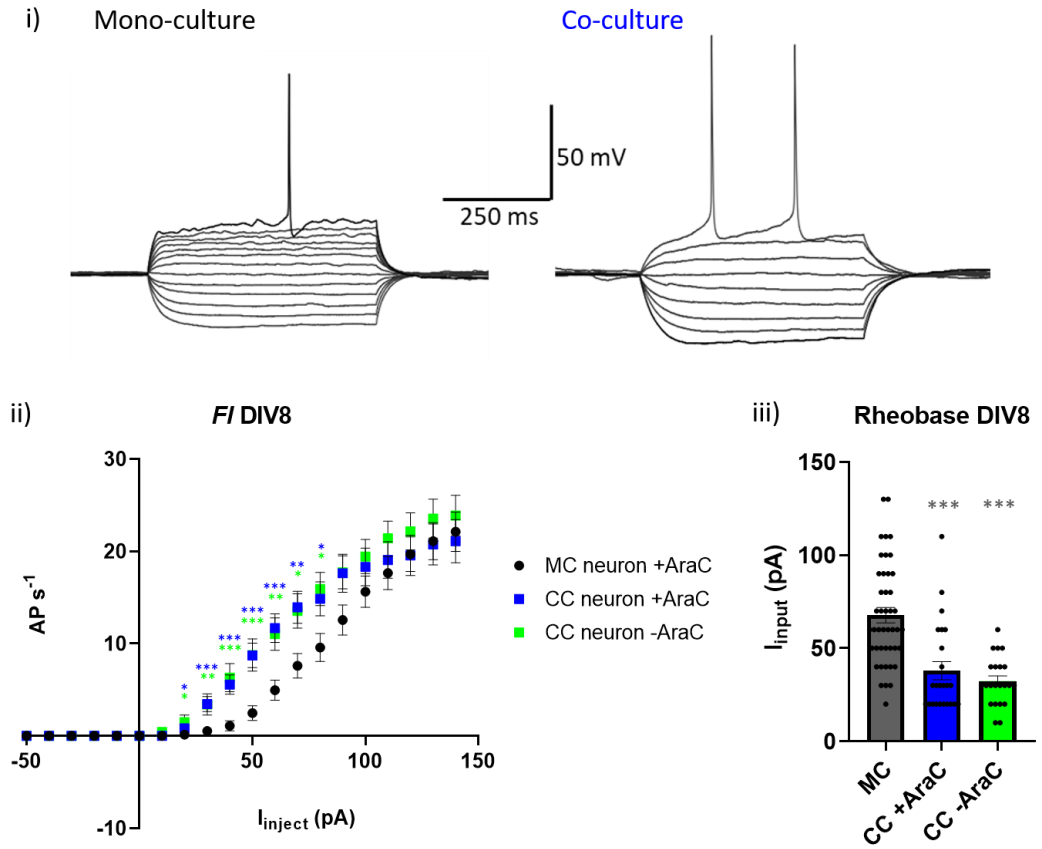


Figure 4.4: Neuronal excitability is increased in the presence of astrocytes

i) Example traces of the frequency-input protocol run on MC and CC cortical neurons, showing the rheobase input step. Neurons were current-clamped at -60 mV, and a series of current injections were applied in successive 10 pA increments (starting from -50 pA injections). Less current was required to be injected into CC neurons to elicit an AP. Of note, the after-hyperpolarisation current appears to be greater in MC neurons than CC neurons. ii) The *FI* curve is leftward shifted in the presence of astrocytes. This corresponds with a significant decrease in the current (I_{inject}) required to elicit an action potential (the rheobase), as seen in iii ($p < 0.001$ for both CC +AraC and CC -AraC compared to MC, LME ANOVA, df = 79). There was no difference between the two CC conditions for any I_{inject} point in the *FI* relationship nor in the rheobase, showing AraC treatment itself is not affecting the observed results. Cells were obtained from 9 independent culture batches.

4.5.1 Effects of astrocytes on neurons are not specific to species

One question arising from the experimental set-up using mixed species (mouse astrocytes and rat neurons) is whether the effect of astrocytes on neurons is due to an effect of different species on gene expression, rather than different cell type. The results of the labs' RNA-sequencing on mouse neuron/mouse neuron plus mouse glia preparations shown in appendix *Figure A6* suggest that this is not the case, as *Kcnj* expression is likewise reduced in glia containing samples. However, to address this concern, I created a same species set-up, using mouse cortical neurons grown on mouse astrocytes. As mouse primary neurons typically develop slightly faster than rat neurons, I additionally created samples of MC and CC mouse neurons that had been incubated in TTX for 48 hours prior to recording to control for any homeostatic masking of differences in properties due to activity that may occur. At DIV8 there was a significant depolarisation in RMP between in CC +TTX treated cells compared to MC +TTX, as well as a slight but insignificant increase in R_M (*Fig A9 i-ii*). In terms of excitability, activity-deprived CC neurons had a significantly lower rheobase than TTX-treated MC mouse neurons (*Fig A9 iii*). These results, combined with same-species RNA-sequencing, suggest the effects of astrocytes on neuronal properties are not due to an interaction of different species, as mouse neurons also benefit from an increase in excitability when grown with mouse astrocytes.

4.6 Blocking K_{IR} in mono-culture mimics the effects of astrocytes on intrinsic neuronal properties

The observed changes in membrane properties and excitability are consistent with a decrease in K_{IR} expression in cortical neurons. I next decided to test whether directly altering K_{IR} activity in cortical neurons was able to change the membrane properties and excitability of cells, to confirm a link with K_{IR} expression to the observed changes in intrinsic properties.

To do this, I grew MC neurons, recorded their properties, acutely applied inhibitors of K_{IR} channels, and then re-recorded their properties in the presence of reduced K_{IR} activity. The RMP and R_M were recorded immediately prior to drug application, and again after 5 minutes of drug wash-on. To begin with I used a combination of the $K_{IR}3.1$ antagonist tertiapin-Q (TQ) and the $K_{IR}2.3$ antagonist ML133. Initially I trialled concentrations that would fully block $K_{IR}3.1$ and 2.3,

which induced a strong change in RMP, R_M and rheobase, but resulted in the cells entering a depolarising block and being unable to fire more than one AP regardless of input current. I then reduced the concentrations of both drugs down to the reported IC_{50} levels for both $K_{IR}3.1$ and 2.3, reasoning that this would result in a partial block of their activity, akin to the partial downregulation (but not complete abolishment) of expression seen in CC.

Application of TQ and ML133 at these lower concentrations resulted in a slight but insignificant depolarisation in the RMP (*Fig 4.5 ii*: mean = -52.5 ± 2.8 and 49.6 ± 2.6 mV, n = 9, before and after drug application, respectively), along with a significant increase in the R_M (*Fig 4.5 iii*: mean = 385.8 ± 45.7 and 516.9 ± 61.5 MΩ, n = 9, before and after drug application, respectively). Along with these changes in membrane properties following $K_{IR}3.1$ and $K_{IR}2.3$ inhibition was an associated increase in excitability, with a leftward shift in the *FI* relationship and a significant decrease in the rheobase (*Fig 4.5 iii-iv*: mean = 68.8 ± 6.0 and 51.1 ± 6.3 pA, n = 9, before and after drug application, respectively).

Next, I looked at the effects of applying the non-specific K_{IR} inhibitor Ba^{2+} to MC neurons. In an attempt to get a partial block of K_{IR} channels at -60 mV, I applied Ba^{2+} at a concentration of 5 μM. There was no effect of applying Ba^{2+} at this concentration on the RMP of MC cortical neurons, however there was a significant increase in their R_M (*Fig 4.6 i & ii*: mean R_M = 451.4 ± 57.1 and 498.8 ± 67.1 MΩ, n = 13, before and after Ba^{2+} application, respectively). As with the specific K_{IR} blockers, the non-specific blocker Ba^{2+} likewise induced a leftward shift in the *FI* relationship and a significant decrease in the rheobase (*Fig 4.6 iii & iv*: mean rheobase = 72.3 ± 6.8 and 54.6 ± 7.3 pA, n = 13, before and after Ba^{2+} application, respectively).

These results demonstrate that reducing K_{IR} activity in MC cortical neurons causes an alteration in their membrane properties and an increase in excitability. The same effect is seen by co-culture with astrocytes, which reduces K_{IR} expression, causing an increase in excitability.

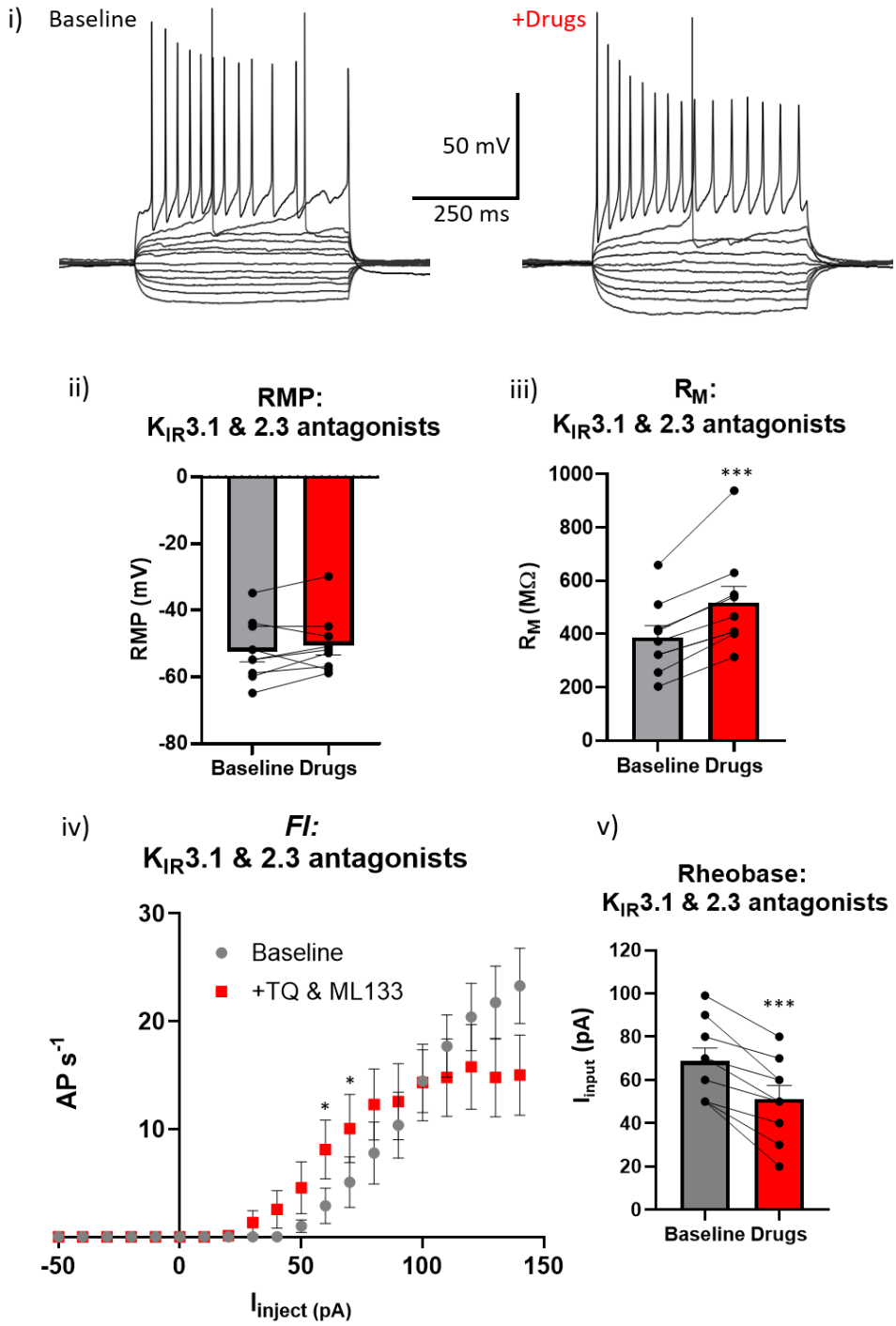


Figure 4.5: Specific K_{IR}3.1 and K_{IR}2.3 antagonism of MC neurons increases excitability

The membrane properties and *FI* relationship (i) were recorded in DIV8 MC rat neurons, with the membrane potential current-clamped at -60 mV for *FI* recordings, before the neuronally expressed K_{IR} channels (K_{IR}3.1 and 2.3) were blocked by acute application of their specific antagonists tertiapin Q (TQ, 15 nM) and ML133 (4 μM). The same properties were then re-recorded in the presence of the drugs. ii) Following drug application there was a slight but insignificant depolarisation in the RMP ($p = 0.1$, n = 9, paired Student's t-test), although a significant increase in R_M was observed (iii; $p < 0.001$, n = 9, paired Student's t-test). iv) and v) There was a leftward shift in the *FI* curve after the application of the antagonists, as well as a significant

reduction in the rheobase ($p < 0.001$, n = 9, paired Student's t-test). Cells were obtained from 3 independent cultures.

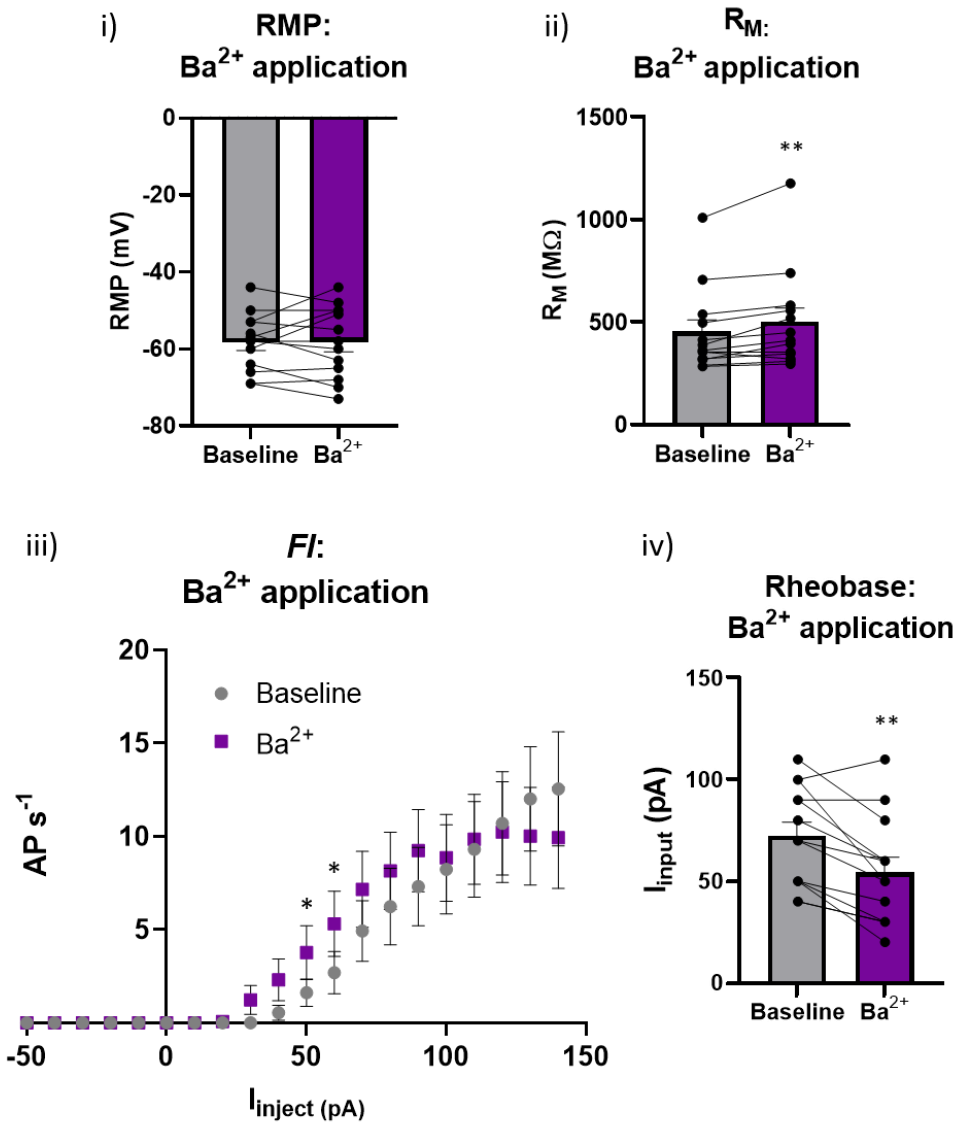


Figure 4.6: Non-specific K_{IR} block with low-dose Ba^{2+} increases excitability of MC neurons

The membrane properties and excitability of MC rat neurons at DIV8 were recorded before the non-specific K^+ channel blocker Ba^{2+} was applied at a low concentration (5 µM) to target the K_{IR} family of channels. After Ba^{2+} wash-on the MC neuron properties were again recorded. i) Application of low dose Ba^{2+} had no effect on the RMP of neurons, but ii) it did significantly increase the R_M ($p = 0.008$, n = 13, paired Student's t-test). iii-iv) Ba^{2+} application caused a leftward shift in the *FI* relationship, as well as a significant decrease in the rheobase of MC neurons ($p = 0.001$, n = 13, paired Student's t-test). Cells were obtained from 5 independent cultures.

4.7 Homeostatic mechanisms mask effects at DIV15, but are recovered by inhibition of activity

Given that K_{IR} expression was observed to increase in MC neurons with development, I hypothesised that the differences between MC and CC properties at a later developmental time point would be enhanced. To investigate this, I maintained MC and CC cortical neurons for two weeks, recording their intrinsic properties and excitability at DIV15, a week later than before. Unexpectedly, at DIV15 there was no change in R_M with CC (*Fig 4.7 i*: mean = 360.1 ± 28.1 and 410.2 ± 44.1 MΩ, n = 24, 25, for MC and CC neurons, respectively, from 5 independent cultures), and only a slight but insignificant depolarisation in RMP (*Fig 4.7 ii*: mean = -50.1 ± 1.9 and -45.8 ± 1.9 mV, for MC and CC, respectively; $p = 0.06$, LME ANOVA). There was also no significant shift in the *FI* relationship, nor a reduction in rheobase, between MC and CC neurons (*Fig 4.7 iii & iv*).

Neuronal properties, including both excitability and miniature EPSCs (mEPSCs), are known to undergo activity dependent homeostatic plasticity, with activity deprivation for 24-48 hours leading to an increase in these activity markers (Echegoyen et al., 2007, Lazarevic et al., 2013, Rich and Wenner, 2007). Therefore, one explanation for the lack of observed difference between MC and CC neurons may be due to an activity dependent regulation in K_{IR} expression. If CC neurons are more active, their functional K_{IR} expression may be increased in a homeostatic mechanism to reduce their excitability. Conversely, the functional expression of K_{IR} channels in MC may be decreased to increase their excitability; however, if this were the case then this could argue against astrocytic regulation of K_{IR} expression.

To investigate if homeostatic mechanisms were masking a difference in K_{IR} expression between MC and CC cortical neurons I incubated both MC and CC neurons in the presence of 300 nM TTX for 48 hours (from DIV13) and then recorded their intrinsic properties following TTX washout. After activity deprivation a significant difference in R_M emerged in neurons grown in the presence of astrocytes (*Fig 4.7 i*: mean = 363.6 ± 27.0 and 594.8 ± 76.5, n = 22 & 23, for MC +TTX and CC +TTX, respectively, from 4 independent cultures). There was also a significant increase in R_M

between control CC neurons and CC +TTX treated cells, but no difference between control MC and MC +TTX.

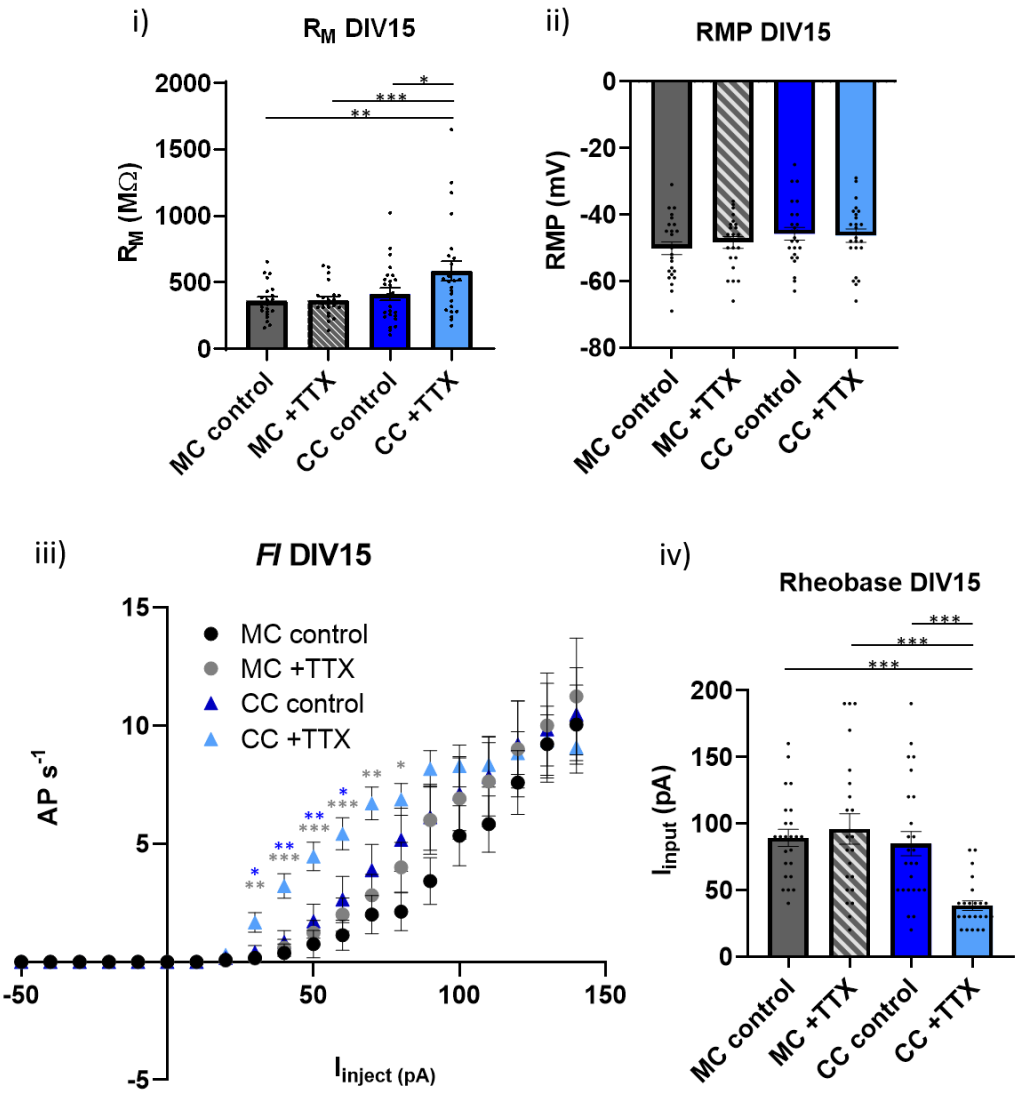


Figure 4.7: At DIV15 the intrinsic properties of CC neurons, but not MC neurons, are activity regulated, masking the effect of astrocytes

The membrane properties and excitability of rat cortical neurons were investigated a week later in development (DIV15). Under normal culture conditions there was no difference in any of the properties between MC and CC neurons at this later timepoint (i-iv). However, 48 hours treatment with the Na_V-channel blocker TTX (300 nM) to block neuronal activity saw a re-emergence of the differences seen at DIV8: i) a significant increase in R_M specifically in CC +TTX ($p = 0.003$, $p < 0.001$, & $p = 0.015$ compared to MC, MC +TTX and CC, respectively, LME ANOVA, df = 82) iii) There was a leftward shift in the FI curve in CC treated with TTX (***** = significance between CC control and CC +TTX, * = significance between MC +TTX and CC +TTX) and iv) a significant reduction in the rheobase only in the CC +TTX condition ($p < 0.001$, LME ANOVA, df = 82). There was no effect of TTX treatment on MC neurons for any property. Cells were recorded from at least 4 independent culture batches.

When I measured the excitability, I found a significant leftward shift in the *FI* relationship of TTX treated CC cells, both from TTX treated MC cells, and from control CC cells (*Fig 4.7 iii*), along with a significant decrease in the rheobase (*Fig 4.7 iv*: mean = 89.1 ± 6.4, 95.9 ± 11.4, 84.8 ± 9.1 and 38.3 ± 3.8 pA, for MC control, MC +TTX, CC control and CC +TTX, respectively). Additionally, a quantitative reverse transcription PCR (RT-qPCR) to investigate the relative expression of *Kcnj3* (K_{IR}3.1) between these four conditions at DIV15 was run by Dr P. Hasel (appendix *A5 i*). At the level of gene expression there was still a reduction in *Kcnj3* expression in control CC neurons compared to MC at DIV15. However, following activity deprivation the expression of *Kcnj3* was further reduced in CC neurons, whilst TTX treatment had no effect on *Kcnj3* expression in MC cells. These results were later confirmed by RNA-seq of DIV15 MC and CC rat neurons ± TTX treatment for the K_{IR} family of genes as a whole (appendix *A5 ii*).

Of note, previous lab RNA-sequencing on the effects of activity on mouse neuronal gene expression showed less *Kcnj3* and *Kcnj4* expression in mixed mouse cells compared to purified neuronal cells, and this expression was further downregulated following TTX treatment, whilst being upregulated following 24 hours of enhanced activity by bicuculline treatment in mixed cell preparations (appendix *A6*). Although not able to distinguish between neuronal and non-neuronal K_{IR} expression in the mixed preparation, these findings further suggest that K_{IR}3.1 and K_{IR}2.3 undergo homeostatic regulation in the presence of astrocytes.

I've shown in this section that although there is significantly less K_{IR}3.1 expression in CC neurons, by DIV15 there is no significant difference in functional measures. However, activity deprivation for 48 hours unmasks the effects of astrocytes on neurons, with TTX treated CC neurons displaying significantly greater excitability. This shows that in the presence of astrocytes K_{IR} expression is activity-regulated, allowing for homeostatic plasticity. It is interesting to note that this form of homeostatic regulation is not seen in MC neurons.

4.9 Protein levels of $K_{IR}3.1$ are decreased in CC neurons

Up until now I have shown a change in K_{IR} gene expression and a change in the functional properties of neurons. I next wanted to confirm whether there was an associated decrease in K_{IR} protein levels in CC neurons. I ran western blots of samples of MC, MC +TTX, CC & CC +TTX treated rat cortical neurons, collected on DIV8 and at DIV15, and tested for $K_{IR}3.1$ expression. The control protein was required to be both neuronally specific (i.e. not expressed in astrocytes), and relatively unregulated by activity. To this end calmodulin was selected, due to its predominant expression in neurons, and the little effect of activity on its expression.

An example blot is shown in *Figure 4.8 i*, where the $K_{IR}3.1$ protein band in CC conditions at both DIV8 and DIV15 is clearly less than in MC. A summary of the expression levels of $K_{IR}3.1$ relative to DIV8 MC neurons, normalised to calmodulin, is shown in *Figure 4.8 ii*. The relative expressions of $K_{IR}3.1$ in both CC control and CC +TTX conditions at DIV8 and DIV15 were significantly lower compared to their MC counterparts. Unexpectedly, there was no significant difference in the relative protein levels of $K_{IR}3.1$ between control and activity-deprived CC neurons at either DIV8 or DIV15.

The decrease in gene expression of $K_{IR}3.1$, and associated change in functional properties, in neurons due to co-culture with astrocytes is supported by a corresponding decrease in protein levels of K_{IR}. However, the activity regulation seen in CC neurons at the gene expression and functional level is not observed at the protein level after 48 hours.

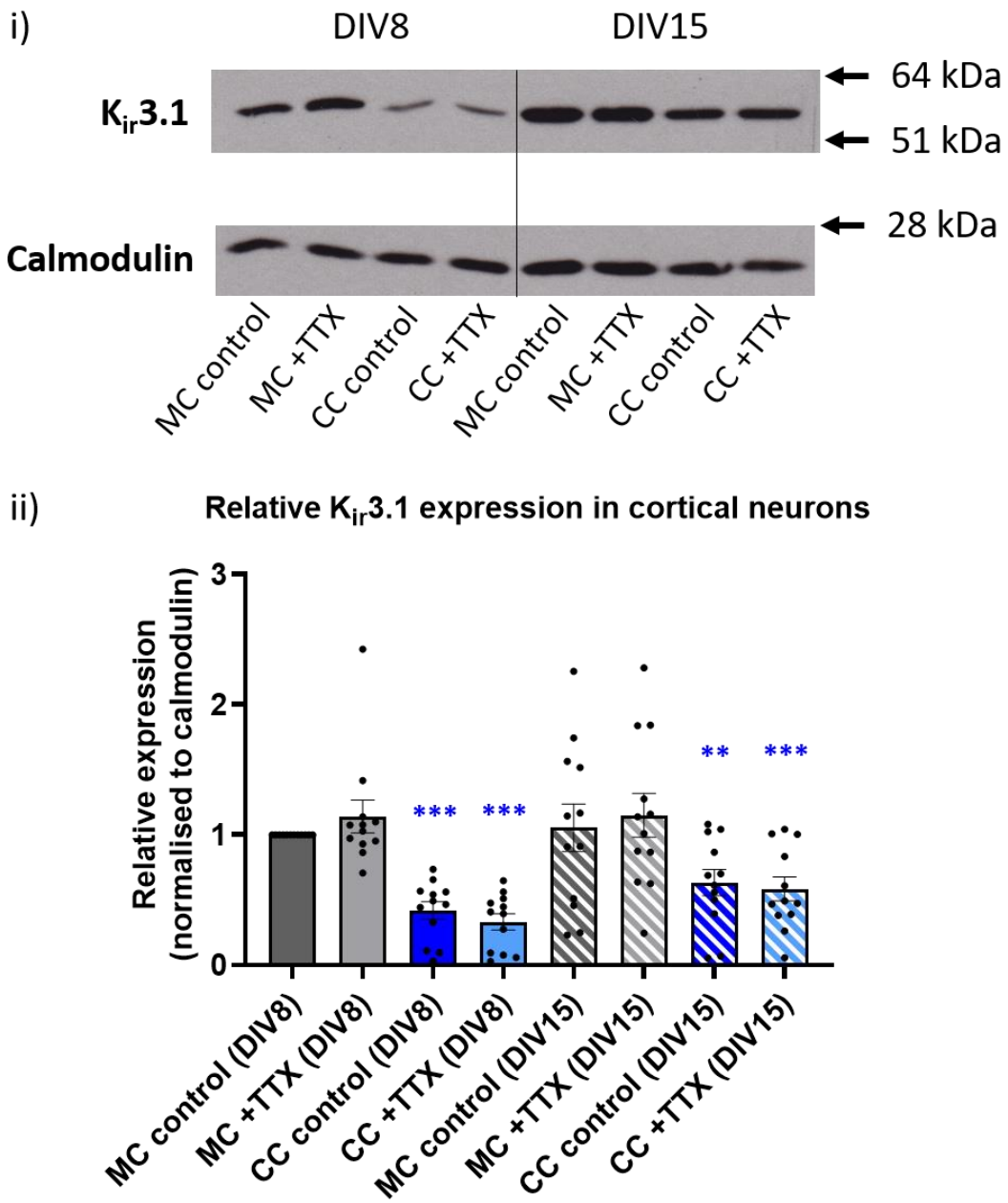


Figure 4.8: Neuronal $K_{IR}3.1$ protein levels decrease with astrocyte co-culture

Protein levels of $K_{IR}3.1$ in cortical rat neurons relative to MC at DIV8, normalised to calmodulin. i) Example western blot stained for $K_{IR}3.1$ and calmodulin. The bands for $K_{IR}3.1$ are clearly stronger in all MC conditions compared to their counterpart CC conditions. ii) At DIV8 protein levels in CC control and CC +TTX are both significantly lower than MC control and MC +TTX ($p < 0.001$ for both, LME ANOVA, df = 77), but there is no difference between CC control and CC +TTX treatment. At DIV15 again there is a significantly less $K_{IR}3.1$ protein in CC relative to MC ($p = 0.005$), and CC +TTX relative to MC +TTX ($p < 0.001$), although there is no difference between CC control and CC +TTX treatment on protein levels of $K_{IR}3.1$ treatment. Sample n = 9 independent cultures.

4.10 An astrocyte secreted molecule is responsible for the change intrinsic properties

Having shown that astrocytes decrease K_{IR} expression in neurons, and that this leads to an associated increase in excitability, I next sought to find the pathway behind this astrocyte-induced reduction in K_{IR}. This would then allow me to confirm that interacting with this pathway both changes K_{IR} expression and alters neuronal intrinsic properties.

To rule in or out an astrocyte secreted factor in inducing K_{IR} expression changes, I investigated the effects of astrocyte conditioned media (ACM) on neurons. Under this set-up, mouse astrocytes are grown in parallel to rat MC neurons, with astrocytes being kept in standard neuronal feeding media (1% NBA). On each day MC neurons were fed (starting with DIV0), half the cells were fed with normal media, and the other half were fed with media that had been collected from the mouse astrocytes (astrocyte conditioned media, ACM).

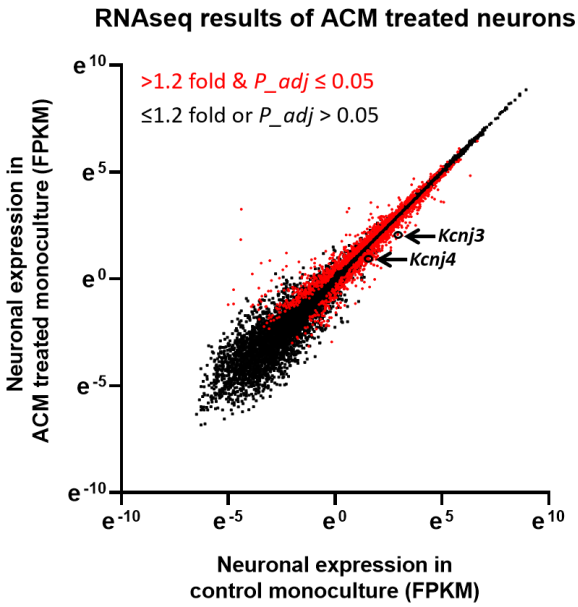


Figure 4.9: Change in mono-cultured cortical neuronal gene expression after three days of astrocyte conditioned media treatment

RNA-seq results of the effects of feeding MC neurons with ACM. MC rat neurons were either fed with standard 1% NBA +AraC or with ACM collected from astrocytes and supplemented with AraC. Although the effect of ACM treatment is not as strong as compared to direct co-culture, there were a total of 1,710 genes significantly up or downregulated >1.2-fold (compared to 1,960 >1.5 fold with co-culture). This included the genes for the K_{IR}3.1 and 2.3 channels (*Kcnj3* and *Kcnj4*), suggesting an astrocyte secreted factor is responsible for the regulation of their expression. Data courtesy of Dr P. Hasel (experimental design and sample preparation) and Drs O. Dando and X. He (bioinformatic processing and analysis).

To begin with, samples from control and ACM treated MC rat neurons were collected, prepared, and sent off for RNA-sequencing (RNA-sequencing sample preparation done by Dr. P. Hasel). The results of this sequencing are shown in *Figure 4.9*. ACM treatment of cortical neurons induced significant changes (with >1.2 fold change) in expression in ~13.5% of genes. This is compared to 14.9% of genes that were seen to change by direct co-culture by >1.5 fold.

Although the effects of ACM were slightly milder than co-culture, the expression of *Kcnj3* ($K_{IR}3.1$) and *Kcnj4* ($K_{IR}2.3$) were both significantly downregulated in MC neurons by ACM treatment (appendix *A7*). This indicates that it is an astrocyte secreted molecule that is triggering the decrease in neuronal K_{IR} expression.

If the decrease in K_{IR} is leading to the alteration in membrane properties and the increase in excitability seen in CC neurons, then ACM treatment of MC neurons should also lead to an increase in excitability, given that it reduces K_{IR} expression. To test this, I recorded from standard MC rat neurons, or MC neurons that had been fed with ACM.

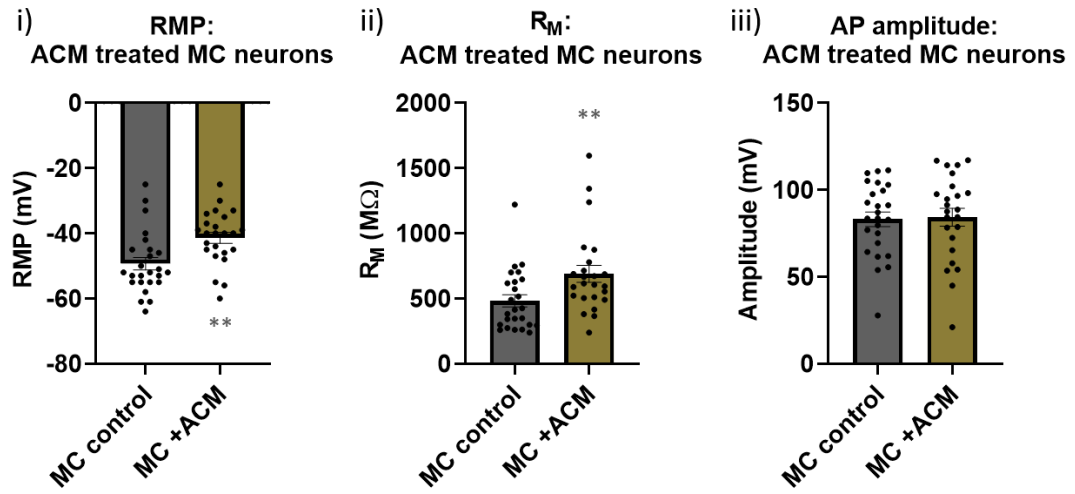


Figure 4.10: Resting membrane potential and membrane resistance of MC rat neurons are affected by ACM treatment

ACM treatment of MC rat neurons has the same effect on membrane properties as direct co-culture with mouse astrocytes. i) RMP was significantly depolarised by ACM treatment ($p = 0.003$, LME ANOVA, df = 44). ii) Membrane resistance of MC neurons was significantly increased by ACM treatment ($p = 0.009$, LME ANOVA). Unlike direct co-culture, there was no effect of ACM on AP height. Cells were obtained from 4 independent cultures.

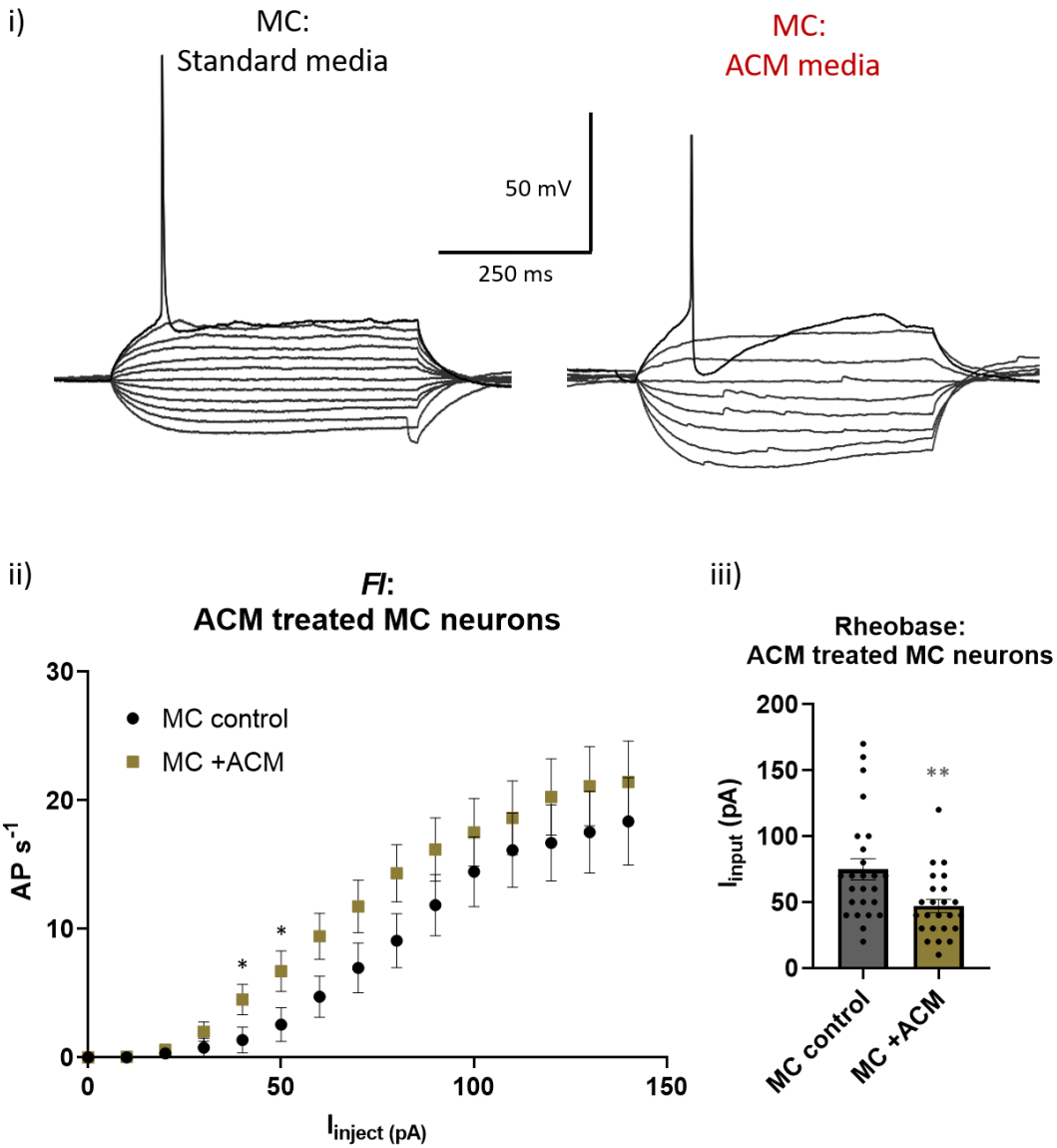


Figure 4.11: Neuronal excitability in DIV8 cells is increased by ACM treatment

Treatment of MC rat neurons with ACM increased excitability at DIV8. i) Example *FI* traces for MC rat neurons grown in regular media or in the presence of ACM at DIV8. ii)The *FI* curve is shifted to the left in MC neurons treated with ACM, requiring less input current to fire action potentials. iii) The rheobase of ACM treated MC neurons was significantly lower, corresponding to their increased excitability (p = 0.004, LME ANOVA, df = 44). Cells were obtained from 4 independent cultures.

As with direct co-culture, ACM treatment of MC neurons caused a significant depolarisation in the RMP (*Fig 4.10 i*: mean = -49.3 ± 1.9 and -41.3 ± 1.7 mV, n = 25 and 24, for control and ACM, respectively, from 4 independent cultures), along with a significant increase in the R_M (*Fig 4.10 ii*: mean = 482.1 ± 46.4 vs 689.2 ± 64.5 MΩ). Unlike direct co-culture, there was no effect of ACM on the action potential amplitude (*Fig 4.10 iii*).

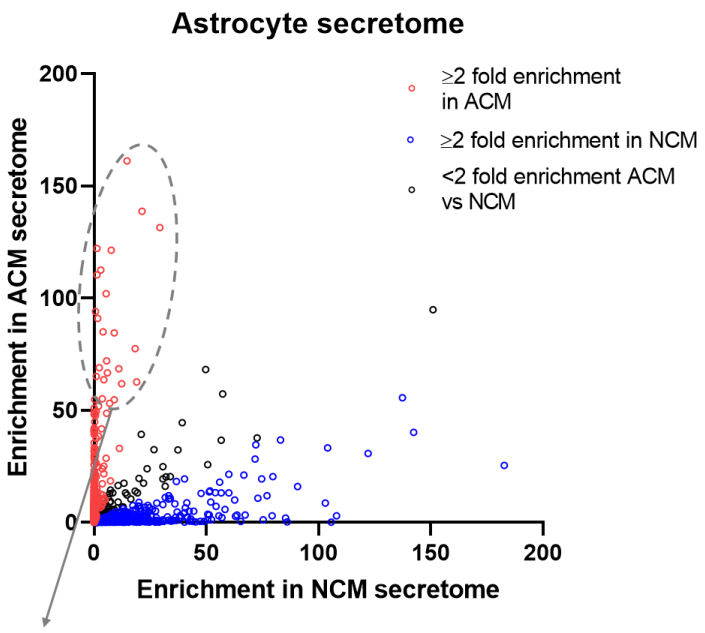
Having established that ACM is able to induce the same changes in membrane properties associated with a decrease in K_{IR} induced by astrocytes, I then looked to the excitability. I found that ACM treatment likewise induced a leftward shift in the *FI* relationship, along with a significant decrease in the rheobase (*Fig 4.11 i & ii*: mean = 74.8 ± 8.1 and 47.1 ± 5.0 pA, n = 25 and 24, for control and ACM, respectively, from 4 independent cultures).

I've shown in this section that an astrocyte secreted molecule induces the decrease in cortical neuronal K_{IR} expression. I've further shown that ACM treatment of MC neurons is likewise able to alter neuronal properties and increase their excitability. This is consistent with an astrocyte-induced reduction in neuronal K_{IR} expression causing alterations in membrane properties, leading to an increase in excitability.

4.11 Secretomics of ACM reveals list of astrocyte released proteins

To narrow down a list of candidate molecules secreted from astrocytes, I prepared and sent off ACM samples for mass spectrometry analysis. Alongside, I also sent off neuronal conditioned media samples (NCM). Mass spec analysis detected 1,478 different types of proteins in the ACM. Of these, 801 were expressed >2 fold compared to proteins detected in NCM. Of the remaining proteins detected in ACM, 560 were enriched by >2 fold higher in NCM samples. Only 117 of the proteins detected had <2-fold difference in enrichment between ACM and NCM. As can be seen in *Figure 4.12 (i)*, a large proportion of proteins detected in ACM are specific to ACM and are not secreted by neurons. The top 25 proteins enriched in ACM are given in *Figure 4.12 (ii)*. Amongst these proteins are the previously mentioned synapse promoting SPARCL1 (Hevin) and its opposing/anti-synapse promoting counterpart, sparc. Also of note is an abundance of apoE in ACM, given that astrocytic cholesterol carried by apoE was hypothesised to increase neuronal activity (Mauch et al., 2001). A full list of enriched ACM proteins is given in appendix *Figure A8*.

i)



ii)

Top 25 proteins enriched in ACM	Gene	ACM expression	ACM:NCM enrichment
Cystatin-C	*Cst3*	423.7	7.5
Apolipoprotein E	*Apoe*	161.2	11.0
Vimentin	*Vim*	138.7	6.5
Neurocan core protein	*Ncan*	131.5	4.5
Pigment epithelium-derived factor	*Serpinf1*	122.2	91.6
Complement C3	*C3*	112.5	37.5
Alpha-2-macroglobulin-P	*A2mp*	110.3	82.8
SPARC	*Sparc*	102.0	19.1
Fibronectin	*Fn1*	94.0	141.0
Insulin-like growth factor-binding protein 2	*Igfbp2*	91.0	54.6
Clusterin	*Clu*	85.0	21.3
Insulin-like growth factor-binding protein 5	*Igfbp5*	84.5	9.4
Cadherin-2	*Cdh2*	77.5	4.2
Cathepsin B	*Ctsb*	72.0	12.7
Cathepsin L1	*Ctsl*	69.0	29.6
Alpha-actinin-4	*Actn4*	68.5	6.2
Beta-2-microglobulin	*B2m*	66.7	11.1
Cathepsin D	*Ctsd*	65.2	65.2
Metalloproteinase inhibitor 2	*Timp2*	62.7	3.3
SPARC-like protein 1	*Sparcl1*	61.8	5.0
Moesin	*Msn*	55.2	15.0
Complement C4-B	*C4b*	54.8	164.5
Transgelin	*Tagln*	54.7	6.1
Nucleobindin-1	*Nucb1*	53.2	7.6
Granulins	*Grn*	51.8	25.9

Figure 4.12: Enrichment of proteins detected in astrocyte conditioned media compared to their enrichment in neuronal conditioned media

i) Typically, proteins tended to be strongly enriched in either ACM or NCM; only a small subset of 117 proteins in ACM were expressed within 2-fold expression difference of neurons (black circles), whereas 801 proteins were enriched >2-fold in ACM compared to NCM (red circles). ii) A list of the top 25 most abundant proteins in ACM that were enriched >2-fold compared to NCM.

This analysis has provided a shortlist of potential signalling proteins that may be responsible for the downregulation of cortical neuronal K_{IR}. My future work will use this list as a guide as I begin a drug screen to determine the pathway responsible.

4.12 Discussion

4.12.1 Summary of findings

In this chapter I have shown that cortical astrocytes control the expression of a wide array of cortical neuronal genes. Included amongst these were the K_{IR} family of genes, which were significantly downregulated by astrocytes. There was a corresponding significant decrease in the protein levels of $K_{IR}3.1$ in neurons grown with astrocytes, along with alterations in membrane properties and ultimately an increase in neuronal excitability after one week of culture. Intriguingly, I found that by the second week of culture the differences in function had largely disappeared between mono- and co-cultured neurons. I showed that this was due to an activity-dependent decrease in excitability specifically in neurons grown with astrocytes, with 48 hours of activity deprivation leading to a re-emergence of the phenotype seen at the earlier time point. Corresponding to this activity-dependent regulation of excitability, there was a decrease in K_{IR} expression in co-cultured neurons following activity deprivation. Interestingly, the properties of cortical neurons, as well as K_{IR} gene expression, grown in the absence of astrocytes were largely unaffected by activity deprivation. I then showed that the signal from astrocytes regulating K_{IR} expression, membrane properties and excitability was a secreted factor. I ran an un-biased mass spec screen for proteins in ACM and NCM, generating a shortlist of candidate proteins enriched in ACM.

4.12.2 Cortical neurons can survive in the absence of astrocytes

In this chapter I have shown that a culture of pure primary cortical rat neurons can be grown and maintained successfully in the absence of glial cells. Applying the drug AraC on the day of plate down and again on DIV4 is sufficient to kill off all rat astrocytes present in the seeded cell suspension, leaving a highly purified culture of cortical rat neurons. Importantly, with just these two doses of AraC, by DIV15 neuronal mono-cultures are still found to be free of astrocytes. On the other hand, astrocytes are found throughout the neurons that were seeded onto established mouse astrocytes, showing that the mouse astrocytes are spared from the effects of AraC on the rat

cells, and remain present in co-culture. Furthermore, at this relatively mature culture timepoint, after the appearance of spines, the cortical neurons were found to be healthy and surviving well. This finding is perhaps surprising, given the importance of astrocytic glutamate clearance for neuronal survival. This could indicate that these neuronal mono-cultures are less active than their *in vivo* counterparts, and their spontaneous release of glutamate is low enough that their few expressed EAATs are sufficient to effectively remove it.

The success of this culture preparation allows me to be able to directly investigate the effects of astrocytes on cortical neurons, rather than using physiologically questionable RGCs as an approximation for these CNS cells. No previous work has directly assessed the impact of astrocytes on cortical neuronal gene expression, but this culture preparation combined with our lab's mixed-species sorting technique, opens up the possibility to address this gap in knowledge (Hasel et al., 2017).

4.12.3 Astrocytes control cortical neuronal gene expression, including the K_{IR} channels

This chapter presents the first report of neuronal gene expression being controlled by astrocytes. I find that nearly 15%, of cortical neuronal genes are significantly up or downregulated by >1.5-fold by cortical astrocytes. Given the extensive involvement of astrocytes in neuronal development and synaptic activity that has been proposed over the past two decades, this degree of astrocytic control was not unexpected. But now for the first time we can appreciate the full extent of the effect of astrocytes on the cortical neuronal transcriptome. Using this information as a guide allows for unbiased hypotheses to be made about how astrocytes control neuronal function.

One of the most frequently proposed ways in which astrocytes control neuronal function is by inducing, increasing and/or promoting excitatory synaptogenesis and maturation. Numerous proteins either excreted or expressed by astrocytes have now been put forward, and are widely accepted, as mediating this astrocyte controlled excitatory synaptogenesis (Allen, 2013). As such, you might expect that a number of the neuronal genes upregulated by astrocytes may be associated with excitatory synapses. Unexpectedly, I found that at the level of gene expression there was no apparent effect of astrocytes on the excitatory synapse components at either DIV8 or DIV15 (see appendix *A3*). This includes both the vesicular glutamate transporter *Slc17a6* (vGlut-2), with astrocytes previously reported to increase and stabilise vGlut-2 synapses, and the "mature" AMPA

receptor subunit, *Gria2* (GluA2), which astrocytes are alleged to promote the expression of (Risher et al., 2014, Blanco-Suarez et al., 2018). Of course, this does not rule out the possibility that there is an increase in protein or surface expression of these synaptic elements in co-cultured cortical neurons, and that the astrocytic control of excitatory synapse formation is independent of the astrocytic control of neuronal gene expression. However, it seems surprising that virtually no excitatory synaptic elements were among the ~15% of astrocyte-controlled neuronal genes. The question remaining then is: if astrocytes are not controlling the expression of these synaptic genes, then what are they controlling?

Interestingly, I found that the expression of the *Kcnj* family of genes was downregulated as a whole in the presence of astrocytes. These genes encode the K_{IR} family of channels, with the two highest expressed subunits in my cortical neurons being *Kcnj3* ($K_{IR}3.1$) and *Kcnj4* ($K_{IR}2.3$), which were also amongst the most downregulated members by astrocytes. This downregulation in gene expression corresponded with a significant decrease in $K_{IR}3.1$ protein level in cortical neurons grown with astrocytes ($K_{IR}3.1$ is relatively specific to our neurons, whereas $K_{IR}2.3$ is expressed in our cortical astrocytes, making it hard to differentiate between neuronal and astrocytic protein in a western blot). Therefore, as K_{IR} channels can have a large impact on the membrane properties of cells, I hypothesised that this astrocytic control of K_{IR} expression may have functional consequences on the cortical neurons and may even be involved in the extensively observed increase in neuronal activity in the presence of astrocytes (Djukic et al., 2007, Hibino et al., 2010, Nägler et al., 2001, Pfrieger and Barres, 1997). I decided to follow up on this hypothesis and focus my investigation on the astrocytic control of neuronal K_{IR} expression.

I note that the downregulation of K_{IR}s in cortical neurons only represents a small fraction of the total effect of astrocytes on neuronal gene expression that were uncovered in this chapter. The bulk of the observed influence of astrocytes over neurons remains to be explored.

4.12.4 Astrocytes control neuronal membrane properties and excitability, purportedly by K_{IR} regulation

Having seen that K_{IR} channels are downregulated in cortical neurons by astrocytes I investigated whether there was a difference in neuronal properties in the presence of astrocytes. In particular, I hypothesised that due to the physiological properties of K_{IR} channels, a reduction in their expression

would be expected to result in an increase in the membrane resistance of neurons, as well as a depolarisation in their RMP away from the K^+ reversal. Both of these outcomes should lead to an enhancement of excitability: higher resistance means less current is required to cross the membrane to depolarise the cell by a given voltage (from Ohm's law), and a depolarisation in RMP means the cell is closer to the action potential threshold, further reducing the current required to induce an action potential. As predicted, I showed that co-cultured DIV8 cortical neurons had higher membrane resistances and more depolarised RMP compared to neurons grown in the absence of astrocytes, along with a corresponding increase in their excitability. These findings supported the presence of reduced K_{IR} expression in co-cultured neurons. Further connecting a decrease in K_{IR} with the change in properties, I demonstrated that by partially blocking K_{IR} in mono-cultured cortical neurons I was able to increase the membrane resistance and enhance neuronal excitability.

This result is particularly interesting as it demonstrates a novel way in which astrocytes control neuronal function that had not been previously described. Furthermore, an increase in excitability may make neurons more likely to fire, and therefore more synaptically active. It is noteworthy that an enhancement in spontaneous activity due to astrocytes has been a consistent finding in many studies, and here I have found a potential and unsuspected mechanism that could explain this observation (Mauch et al., 2001, Pfrieger and Barres, 1997, Allen et al., 2012, Ullian et al., 2001, Christopherson et al., 2005). Peculiarly, the earliest paper demonstrating that astrocytes increase the activity of RGCs *only* explored the role of enhanced excitatory synaptogenesis in this increased activity – they did not investigate whether there was an increase in the excitability of RGCs grown with astrocytes that could also lead to these observations, even while noting that it was a possibility (Pfrieger and Barres, 1997). Ever since, although repeatedly finding that astrocytes increase activity, subsequent studies have neglected to investigate whether neuronal (or RGC) excitability is enhanced by astrocytes. In hindsight, this may have been an oversight.

4.12.5 An astrocyte secreted protein is responsible for cortical neuronal K_{IR} regulation

I wished to uncover the signalling pathway by which astrocytes regulated neuronal K_{IR} expression in order to undertake experiments that could confirm the role of K_{IR} regulation in controlling neuronal excitability. Overall, the majority of astrocytic communication with neurons that has been previously described in literature has been mediated by astrocyte secreted factors, with occasional

exceptions such as astrocytic neuroligin-2 (Stogsdill et al., 2017). Taking the approach of other labs to investigate if an astrocyte secreted factor or physical contact mechanism was behind the downregulation of K_{IR}, I grew cortical neurons in regular media or ACM and recorded these cells' intrinsic properties. Concurrently, my colleague Dr Philip Hasel sent off samples of these cells for RNA-sequencing to explore the cortical neuronal genes regulated by astrocyte secreted factors compared to direct co-culture. I found that ACM was able to virtually replicate the effects of direct co-culture on neuronal membrane resistance, RMP and excitability, suggesting that a factor in ACM downregulates neuronal K_{IR} expression. Happily, when we received the results from the RNA-seq of these ACM treated neurons I found that, as with direct co-culture, ACM treatment did indeed cause a reduction in *Kcnj3* and *Kcnj4* expression. These findings show that unlike the neuronal regulation of astrocytic EAAT expression that I uncovered in *Chapter 3*, astrocytes regulate K_{IR} expression in neurons by a secreted factor.

To further explore what this factor and downstream signalling pathway in neurons could be, I prepared and sent off both ACM and NCM samples for mass spectrometry analysis. Comparing the proteins present in both samples, I generated a list of proteins enriched in ACM over NCM. Convincingly, many of the previously reported astrocyte secreted factors involved in promoting neuronal activity and synaptogenesis were present and enriched in my ACM sample, including thrombospondin-1, glypican-4 and -6, sparc and SPARCL1 (Kucukdereli et al., 2011, Allen et al., 2012, Christopherson et al., 2005). However, one of the most abundantly present proteins enriched in my ACM samples was apoE: although apoE was not found to increase activity, it can carry cholesterol which has previously been observed to increase activity in RGCs (Mauch et al., 2001). In experiments going forward I will need to confirm whether or not it is a protein or other factor (such as cholesterol) in ACM that reduces neuronal K_{IR} expression, for example by protease treatment of the ACM to denature the proteins.

4.12.6 Neurons require astrocytes for activity-dependent homeostatic plasticity of excitability

From the initial RNA-seq data I observed that K_{IR} expression increased in cortical mono-culture neurons from DIV8 to DIV15, which lead me to hypothesise that the effects of astrocyte co-culture on membrane properties and excitability would be even more pronounced by DIV15.

Unfortunately, this initial dataset did not include a DIV15 CC neuron sample, which would have shown that the effects of astrocytes on K_{IR} repression are not as great at this time point. Instead this first became apparent following intrinsic property recordings of DIV15 MC and CC neurons, where I found no significant difference in membrane properties or excitability due to astrocytes. At this point I wondered whether this apparent lack of effect of astrocytes was due to homeostatic processes in the CC neurons induced by an increased level of activity in this condition that caused the effect of astrocytes to be obscured. To find out, I treated both MC and CC neurons with TTX to inhibit activity for 48 hours before recording. I discovered that after activity deprivation there was a pronounced effect of astrocytes on the membrane properties of neurons, uncovering a significant increase in neuronal excitability in these co-cultured cells. Most interestingly of all, activity deprivation had very little effect in MC neurons. In parallel, Dr Philip Hasel ran an RT-qPCR for *Kcnj3* ($K_{IR}3.1$) for these four conditions, which supported my functional findings, showing a decrease in *Kcnj3* expression following TTX treatment only in CC neurons. A subsequent RNA-seq data set (this time including DIV15 CC neurons) further supported these findings (see *Appendices A4 & A5*).

Previous literature had established a role for astrocytes in mediating both LTP and LTD forms of plasticity, with one report additionally demonstrating astrocytic involvement in homeostatic plasticity of synaptic strength (Stellwagen and Malenka, 2006, Henneberger et al., 2010, Min and Nevian, 2012, Navarrete et al., 2012, Navarrete et al., 2019, Adamsky et al., 2018). My results in this chapter suggest that astrocytes are also involved and/or required for the homeostatic regulation of cell excitability. This is the first report of astrocytes' involvement in this form of plasticity, and if confirmed could position astrocytes' control of plasticity as one of their fundamental functions in the CNS, being involved with all of its flavours.

4.12.7 Limitations and future work

There are still several gaps in this work that need addressing. Firstly, much work still has to be done to identify the signalling pathway that drives the astrocyte induced decrease in K_{IR} expression, starting with confirming if it is a protein or other factor present in ACM that is responsible for the induction. Once the factor and mediating pathway have been established, I will be able to better

address the remaining holes in this body of work, with the potential to extend the work into an *in situ* or *in vivo* model to investigate the relevance beyond this culture preparation.

To begin with I will confirm whether it is a protein or other secreted factor that mediates the regulation of K_{IR}, by treating ACM with protease to degrade the proteins present before applying to MC neurons. If there is still an effect of this protein depleted ACM on K_{IR} expression the finger will be largely pointing towards a non-protein factor such as cholesterol, but if not then a protein detected from my mass spec results will be more likely. Next, I will run an RT-qPCR drug screen on ACM treated MC neurons treated with a variety of different inhibitors targeting pathways/receptors known to be activated by the top protein/non-protein candidates, as applicable. Once I have found a likely suspect, I will then apply the identified factor to MC neurons to see if said factor can repress K_{IR} expression. If this is successful, I will record the electrophysiological properties of MC neurons treated with the factor and CC neurons treated with antagonists for the suspected factor's receptors. If these recordings are successful, I will then seek to develop a mouse model with conditional astrocytic knockout of the suspected factor, or neuronal knockout of the target receptor/pathway in order to explore the relevance of this signalling pathway between astrocytes and neurons in an *in vivo* setting.

Secondly, as yet I have not demonstrated beyond doubt that the astrocyte induced decrease in neuronal K_{IR} mRNA and protein is responsible for the astrocyte mediated changes in neuronal membrane properties and excitability. First and foremost, I have not directly measured whether K_{IR} currents themselves are reduced in the presence of astrocytes, I have only measured the secondary anticipated changes in membrane properties. Therefore, to begin with I will measure the K_{IR} specific currents in MC and CC cortical neurons, by running a series of hyperpolarising current injection steps in the absence and presence of Ba^{2+} and/or the specific $K_{IR}3.1/2.3$ blockers tertiapin Q/ML133, and measuring the elicited instantaneous and steady state currents. If there is less functional K_{IR} in CC neurons, then I should observe a smaller drug-sensitive current component. Additionally, I intend to discover the signalling pathway involved in K_{IR} mRNA repression, as described above. I can then investigate if simply by applying the factor identified as being responsible for causing the repression of K_{IR} to MC neurons is able to reduce the K_{IR} currents and alter the intrinsic properties of neurons in a similar manner to astrocytes. I can also then block the neuronal target receptors, or knockdown the expression of the suspected factor in astrocytes with

shRNA (before neuronal plate-down to ensure astrocyte specific removal) to see if this prevents the decrease in K_{IR} expression in CC neurons. Given my results so far, it would be surprising if it did not turn out to be the case that astrocyte reduction of K_{IR} mediates the changes in intrinsic properties, but never-the-less astrocytes do alter the membrane properties and increase excitability of cortical neurons – even in the unlikely scenario that this regulation does not involve K_{IR} functional expression.

Finally, although I have found that neuronal K_{IR} appears to undergo activity-dependent homeostatic regulation, and that astrocytes are required for the homeostatic plasticity of neuronal excitability, I have not explored whether the regulation of K_{IR} drives this form of plasticity, or if some separate mechanism is involved. Before finding the secreted factor, I can begin to investigate this by looking at the functional changes to excitability in DIV15 MC neurons treated with ACM ± TTX. This experiment will allow me to see if astrocytes are actively involved in activity-dependent homeostatic plasticity, or if the emergence of this plasticity in neurons is due to a passive effect of astrocyte factors. If astrocytes actively respond to regulate K_{IR}/excitability in response to changes in neuronal activity, then I should not see an effect of activity deprivation in ACM treated MC neurons on excitability. To further investigate the involvement of K_{IR} in the observed homeostatic regulation in the presence of astrocytes, once I have isolated the astrocyte factor, I can inhibit the neuronal receptor or knockdown the factor in astrocytes to see if this prevents the activity dependent homeostatic regulation of excitability.

4.12.8 Conclusion

In this chapter I have presented the first report of the astrocytic control of cortical neuronal gene expression. This opens up the door for many future investigations on astrocytes' control of neuronal functions, far beyond the scope of this thesis. Here I have begun by demonstrating that astrocytes control the functionally important K_{IR} channels in cortical neurons by a secreted factor, and that astrocytic control leads to an increase in neuronal excitability. I have additionally shown that astrocytes allow these neuronal K_{IR} channels as well as neuronal excitability to undergo activity-dependent homeostatic plasticity. A putative model for the astrocytic regulation of neuronal K_{IR} during development and in response to activity is presented in *Figure 4.13*.

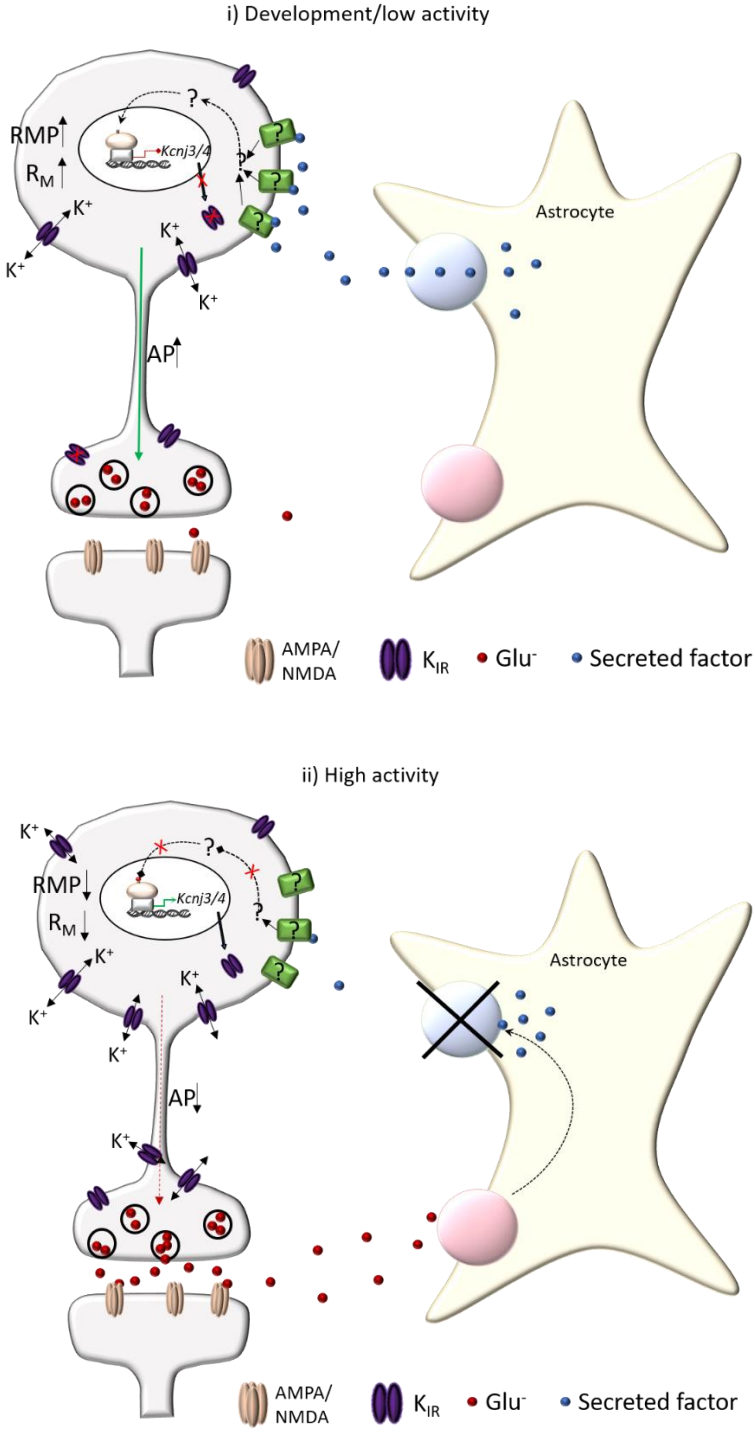


Figure 4.13: Model of astrocytic control of neuronal K_{IR}

i) During development (and low neuronal activity levels) astrocytes secrete a factor that induces a pathway that represses the transcription of *Kcnj*, reducing functional K_{IR} channel expression, leading to a depolarisation in the RMP and an increase in the R_M of neurons. This causes an increase in action potential generation. ii) Under high activity levels a signalling pathway is activated that prevents the release of the astrocytic factor, preventing the repression of *Kcnj* transcription, causing an increase in functional K_{IR} expression, hyperpolarisation of RMP and a decrease in R_M, ultimately reducing AP generation and activity.

From the work in this chapter I hypothesised that neurons grown in the presence of astrocytes would show an increased amount of spontaneous activity, driven by this enhanced excitability. Given the previous literature documenting an effect of astrocytes on excitatory synapse formation, I further hypothesised that an increase in activity might be additionally driving the previously reported effects on excitatory synaptogenesis. I explore these possibilities in the following chapter.

Chapter 5

Cortical neurons do not need cues from astrocytes to form synapses; astrocytes instead enhance network activity and plasticity

Chapter 5 – Cortical neurons do not need cues from astrocytes to form synapses; astrocytes instead enhance network activity and plasticity

5.1 Introduction

In the previous chapter I showed that astrocytes increase the excitability of cortical neurons, by ostensibly decreasing neuronal expression of K_{IR} channels. The question then is, does this change in excitability effect the activity of neurons, and if so how? There is a growing body of literature suggesting that astrocytes increase spontaneous activity, promote synaptogenesis and are required for certain forms of plasticity, including LTP and LTD (Adamsky et al., 2018, Henneberger et al., 2010, Allen, 2013, Pfrieger and Barres, 1997). It is conceivable that alterations in neuronal excitability may interact, or be directly involved, with any of these processes, given that an increase in the global average excitability of cells would be predicted to result in an increase in the activity across the network.

Astrocytes were shown to be able to increase neuronal activity in retinal ganglion cell cultures in the 1990s (Pfrieger and Barres, 1997). The authors noted that by culturing purified RGCs with cortical astrocytes they were able to transform these otherwise rather silent cells into cells displaying robust spontaneous activity (Pfrieger and Barres, 1997). They went on to shown that the astrocytes were able to induce excitatory synapse formation in these prepared RGCs, leading to the hypothesis that astrocytes are required for synaptogenesis (Ullian et al., 2001, Nägler et al., 2001). A body of work has since been built on this, with several astrocyte secreted molecules having been shown to play a role in the excitatory synaptogenesis seen in RGCs (Chung et al., 2015, Baldwin and Eroglu, 2017). Unexpectedly, I did not see an increase in excitatory synapse associated genes in cortical neurons co-cultured with astrocytes, but it is conceivable that the reported synaptogenic effect of astrocytes could be downstream of the increase in excitability: increased excitability may increase activity, and activity may drive synaptogenesis.

A key feature of plasticity is the ability of neuronal networks to alter their responses following stimulation by either increasing or decreasing their responses to future stimuli (Rich and Wenner,

2007, Martin et al., 2000). I found in the previous chapter that co-cultured neurons were able to regulate their expression of K_{IR}, along with their excitability, in response to changing activity levels, whereas mono-cultured neurons were not, suggesting astrocytes are required for homeostatic plasticity of neuronal excitability. Additionally, astrocytes have previously been reported to be required for homeostatic plasticity of synaptic strength (Stellwagen and Malenka, 2006). If this is also true of the cortical neurons in my preparation, then through the combination of these two forms of plasticity astrocytes would be predicted to exert extensive control over the homeostatic regulation of synaptic activity of neurons.

In this chapter I explore these questions. First, I hypothesise that the decreasing K_{IR} levels and increasing excitability of cortical cells grown with astrocytes leads to an increase in spontaneous activity of neurons. Second, I hypothesise that if there is an increase in activity in the presence of astrocytes, I will also see an increase in excitatory synapses. Finally, I hypothesise that there will be a homeostatic increase in synaptic strength resulting in an increase in the network activity of co-cultured neurons in response to activity deprivation, whilst there will be little effect of activity deprivation on mono-cultured neurons. As with *Chapter 4*, for the experiments in this chapter I used rat cortical neurons grown alone in mono-culture (MC) or grown on a bed of mouse astrocytes, referred to as co-culture (CC).

5.2 K_{IR} overexpression blocks spontaneous activity in cortical neurons

To see if K_{IR} expression could alter activity levels in neurons, I first transfected CC cortical neurons with either a globin-GFP plasmid or a K_{IR}2.1-GFP plasmid, in order to generate neurons that overexpress K_{IR}. I then recorded the spontaneous firing of these CC neurons in current-clamp. Globin transfected controls on average fired 55 ± 23 AP per minute (*Fig 5.1 i & ii,* n = 12), whereas K_{IR}2.1 overexpressing neurons only fired an average of 1 ± 1 AP per minute (*Fig 5.1 ii & iii*, n = 12). In fact, most K_{IR}2.1 overexpressing neurons were not observed to fire once over the duration of a 5-10 minute recording. Not only did K_{IR}2.1 overexpression reduce the likelihood of CC neurons spontaneously firing action potentials, it also significantly hyperpolarised their RMP compared to globin transfected controls (*Fig 5.1 iv*: mean RMP = -44.6 ± 2.6 vs -75.5 ± 3.2 mV, n = 12 and 12, for globin and K_{IR}2.1, respectively, from 3 independent cultures). This demonstrates that alterations in K_{IR} expression can alter neuronal activity.

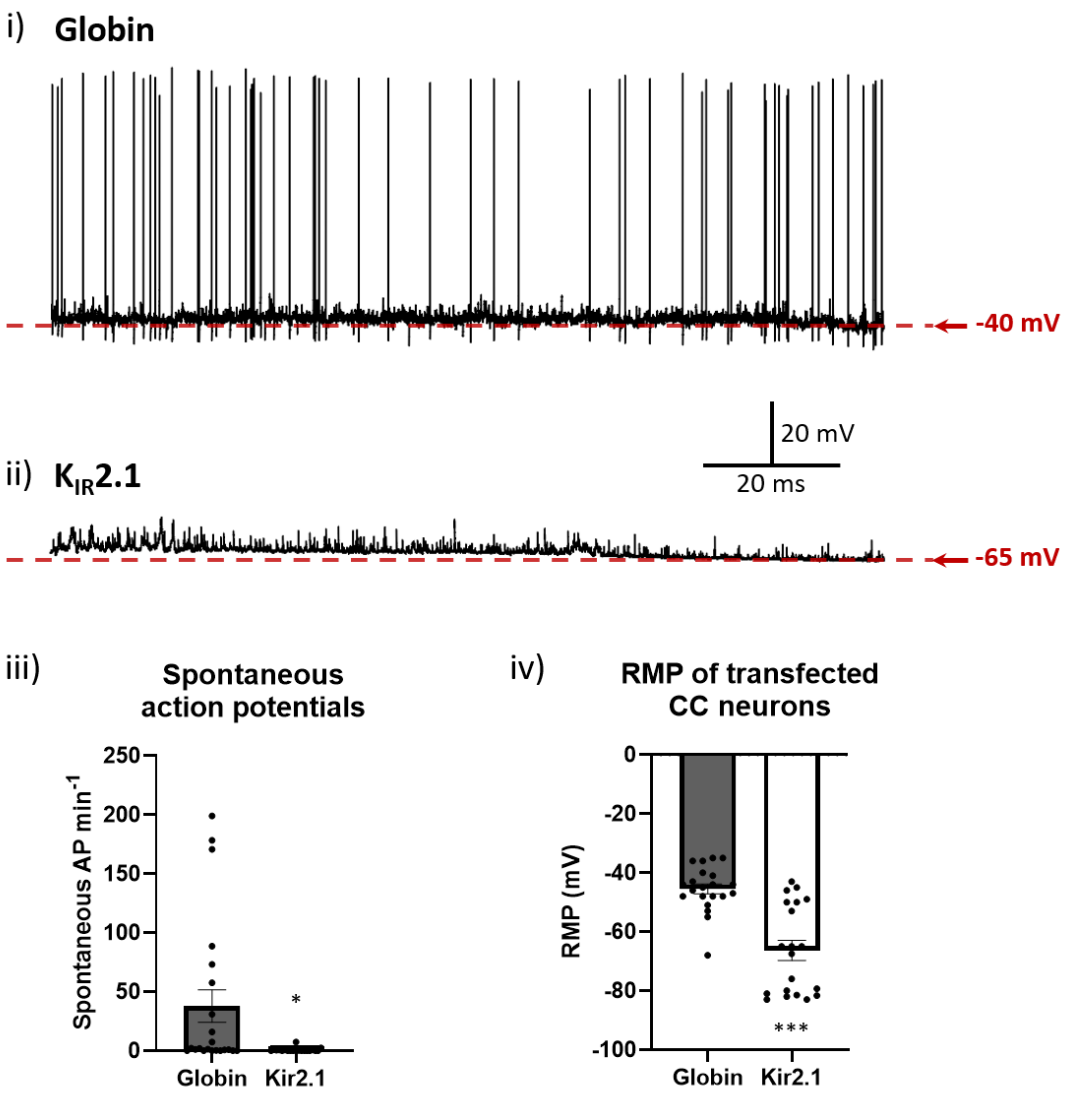


Figure 5.1: $K_{IR}2.1$ overexpression reduces spontaneous activity in co-cultured cortical neurons

Cortical CC neurons were transfected with either a globin-GFP plasmid or a $K_{IR}2.1$-GFP plasmid. The RMP was taken and the spontaneous firing of GFP-positive cells was recorded in current-clamp. i) Example trace of the spontaneous firing of a CC globin expressing cell compared to ii) a $K_{IR}2.1$ positive cell. iii) Transfection with $K_{IR}2.1$ reduced spontaneous firing of cortical CC neurons (p = 0.02, student t-test, n = 12 & 12, for globin and $K_{IR}2.1$, respectively). iv) Transfection with $K_{IR}2.1$ significantly hyperpolarises the RMP of CC neurons compared to control (p < 0.001, Student's t-test, n = 12 & 12 for globin and $K_{IR}2.1$, respectively). Recordings are from 3 independent cultures.

5.3 Spontaneous activity is higher in co-cultured cortical neurons

I next investigated if the spontaneous activity of cortical neurons was higher in CC compared to MC cells. To do this I recorded the cells in voltage-clamp, and measured the spontaneous amplitude, frequency and charge transfer occurring over several minutes.

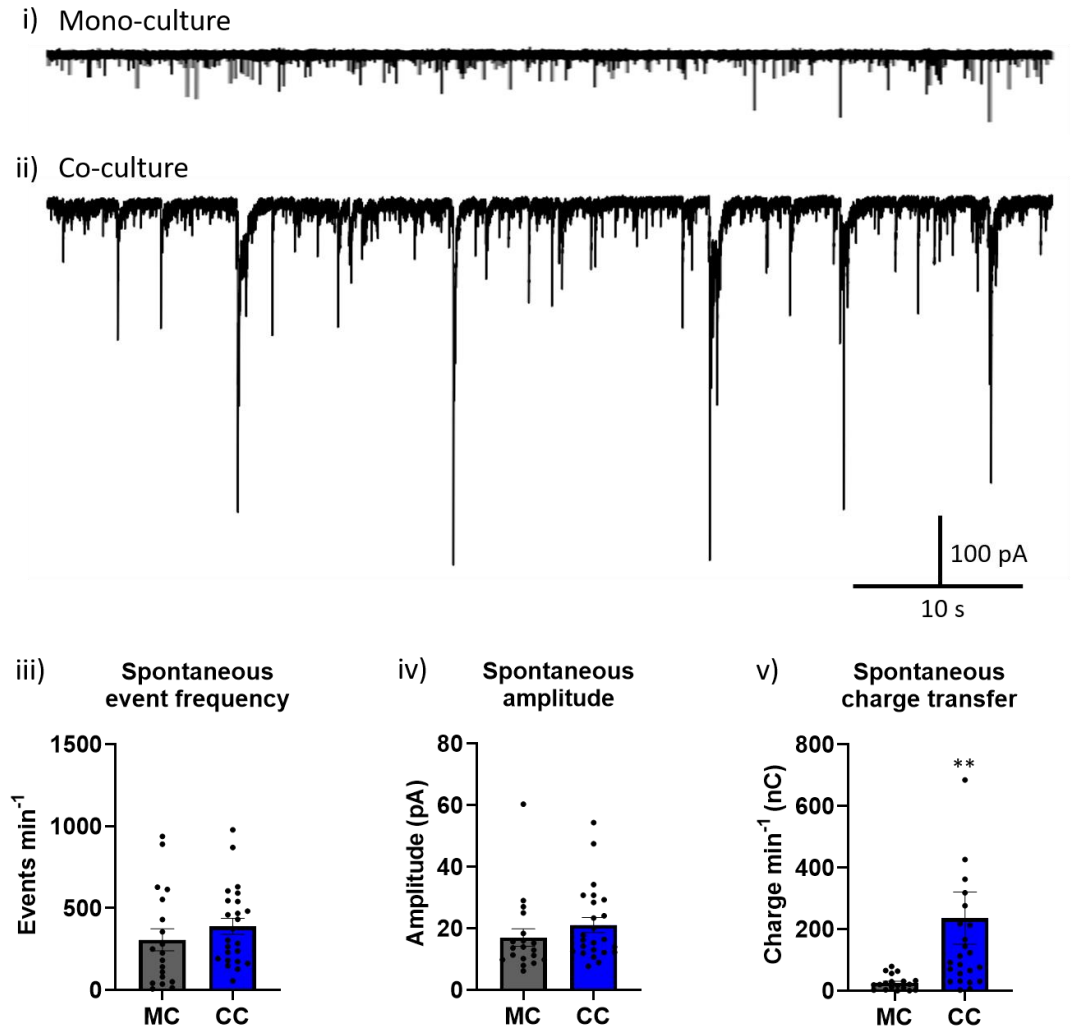


Figure 5.2: Spontaneous activity is greater in co-cultured cortical neurons at DIV8

Example traces of DIV8 i) mono-culture and ii) co-culture cortical neurons, voltage-clamped at -60 mV. Larger "network" burst firing events can be seen in CC compared to MC, which likely result from current generated by voltage-gated Ca^{2+} and Na^{+} channels. iii) & iv) There was no significant difference in frequency or amplitude of events between MC and CC, v) but the observed increase in large network events was represented by significantly greater charge transfer in neurons cultured with astrocytes compared to mono-culture (p = 0.01, LME ANOVA, df = 47, n = 19 & 24, MC & CC). Cells were obtained from 11 independent cultures.

There was a clear increase in the number of spontaneous network events in CC cells compared to MC cells, characterised by large inward current bursts as seen in *Figure 5.2 ii*, which were nearly always absent in MC cells (*Fig 5.2 i*). The increase in network events with CC was not marked by a significant difference in the average amplitude or frequency of events, but instead it can be seen in the significant increase in charge crossing the membrane in CC cells (*Fig 5.2 vi*: mean = 25.8 ± 5.5 and 235.8 ± 85.2 nC min^{-1}, n = 19 and 24, MC and CC, respectively, from 11 independent cultures).

5.4 There is no difference in TTX-*insensitive* (miniature EPSCs) event properties between mono- and co-cultured neurons

As astrocytes increased the activity levels of cortical neurons, I next looked into whether there was an increase in excitatory synapse formation in CC by measuring miniature EPSCs (mEPSC). mEPSC are non-action potential dependent excitatory post-synaptic currents, and their frequency and amplitudes can be used as an indication of the number of established synapses present. Miniature EPSCs can be recorded in voltage-clamp whilst applying the Na_V-channel blocker TTX to prevent action potential firing, and the GABA antagonist picrotoxin (PTX) to eliminate detection of non-action potential dependent release of GABA.

Contrary to my expectations, I saw no difference between mEPSC recordings of MC (*Fig 5.3 i*) and CC (*Fig 5.3 ii*) cortical cells at DIV8. This is seen by a lack of difference in the amplitudes, frequencies and charge transfer between MC and CC cortical neurons (*Fig 5.3 iii-v*: n = 19 and 24, MC and CC, respectively, from 11 independent cultures).

These recordings were done at DIV8 – at this stage in primary rat culture spine formation has yet to begin, and it may be too early to see an effect of astrocytes on excitatory synapse formation. As such I repeated the experiment at DIV15, by which point my rat primary neurons had developed spines, to see if there was an astrocytic induction of excitatory synapse formation later in development.

As was observed at DIV8, there was still no difference in any mEPSC property between MC and CC cortical neurons (*Fig 5.4*; n = 22 and 22, MC and CC, respectively, 7 independent cultures).

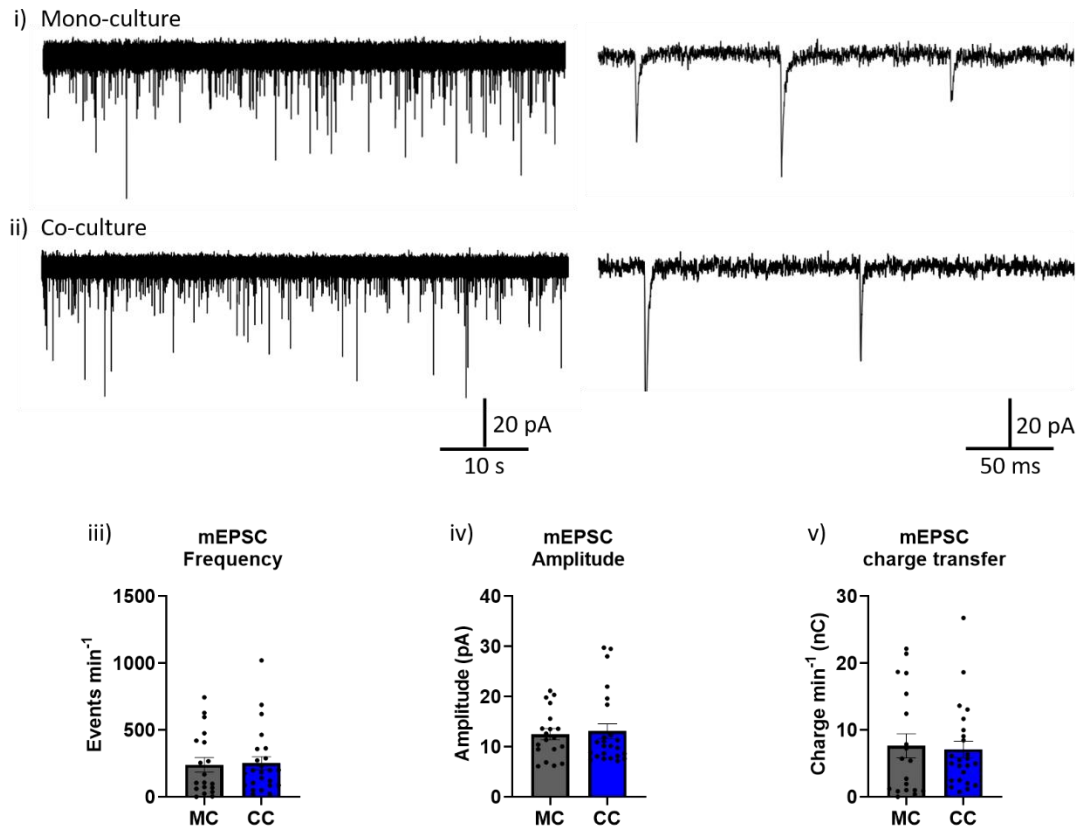


Figure 5.3: TTX-insensitive currents (mEPSCs) in DIV8 cortical neurons are unaltered by astrocytes

Co-culture with astrocytes had no effect on TTX-insensitive mEPSC events. i-ii) Example traces of mEPSC recordings. All cells were voltage-clamped at -60 mV and recorded in the presence of 300 nM TTX and 50 µM PTX. iii-v) Neither the frequency (p = 0.9, LME ANOVA, df = 46), amplitude (p = 0.7) or charge (p = 0.8) of mEPSC events were altered by co-culture with astrocytes. n = 19 & 24 for MC & CC, respectively, 11 independent cultures.

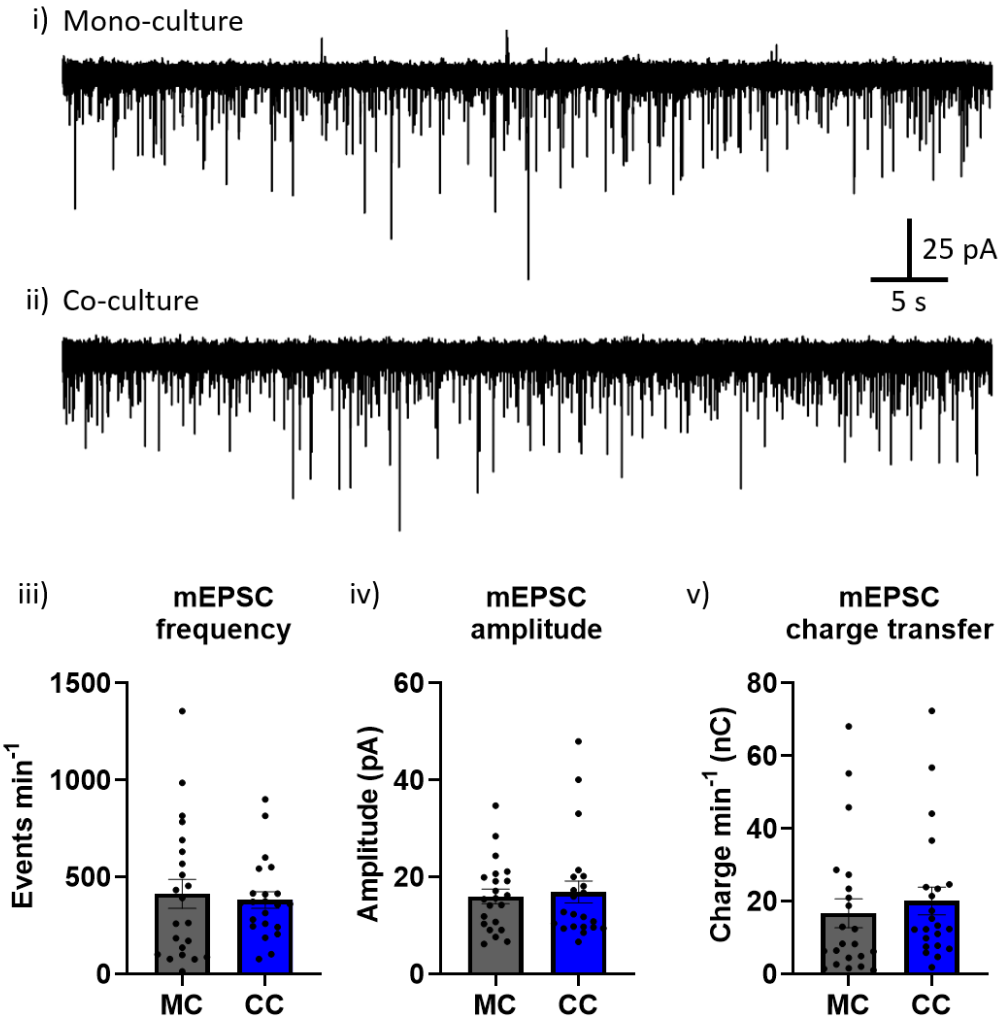


Figure 5.4: TTX-insensitive currents (mEPSCs) in DIV15 cortical neurons are unaltered by astrocytes

At the later developmental timepoint of DIV15, no difference in mEPSCs had arisen between MC and CC cortical neurons. i) & ii) Example mEPSC recordings in MC and CC DIV15 neurons. iii-v) There was no difference in the frequency ($p = 0.7$, LME ANOVA, df = 42), amplitude ($p = 0.8$) or charge transfer ($p = 0.5$) of mEPSC events between MC and CC neurons (n = 22 & 22 for MC & CC, respectively, 7 independent cultures). All recordings were done in the presence of TTX (300 nM) and PTX (50 μM).

5.5 There is no difference in synapse numbers between mono- and co-cultured cortical neurons

Although I saw an increase in activity in CC neurons, I did not find this to be accompanied by an increase in mEPSCs. This suggests that there is no astrocytic promotion of excitatory synapse formation in these cortical cells. To confirm that this was the case, I used immunohistochemistry to visualise the number of excitatory synapses in MC and CC cells.

Due to the density of the cells in the culture preparation, it is hard to identify single dendrites from each other in order to be able to count the synapse numbers along a single branch. To get around this problem I sparsely transfected the cortical neurons with EF-GFP on DIV4, in order to visualise single cells. On DIV15 the cells were fixed and stained for antibodies against the post-synaptic marker Homer-1, the excitatory pre-synaptic marker Synapsin-1, and GFP.

Three separate dendrites, located approximately 100 μm from the soma, were imaged for each GFP-positive cell (*Fig 5.5 i & ii*). Co-localised pre- and post-synaptic markers along these GFP-positive dendrite stretches were manually selected for puncta overlap and their numbers counted. The average number of synapses per 10 μm was then calculated per dendrite and per cell.

There was no difference in the number of excitatory synapses (co-localised pre- and post-synaptic puncta) between DIV15 MC and CC cells (*Fig 5.5 iii*: mean synapses 10 μm^{-1} = 2.76 ± 0.25 and 2.45 ± 0.30 per cell, n = 15 and 14 cells for MC and CC, respectively, from 3 independent cultures). These results support the functional observation of no difference between the mEPSCs recorded in MC and CC cortical cells.

I've shown in the last two sections that my cortical astrocytes are either a) not able or b) not required to promote excitatory synaptogenesis in cortical rat neurons.

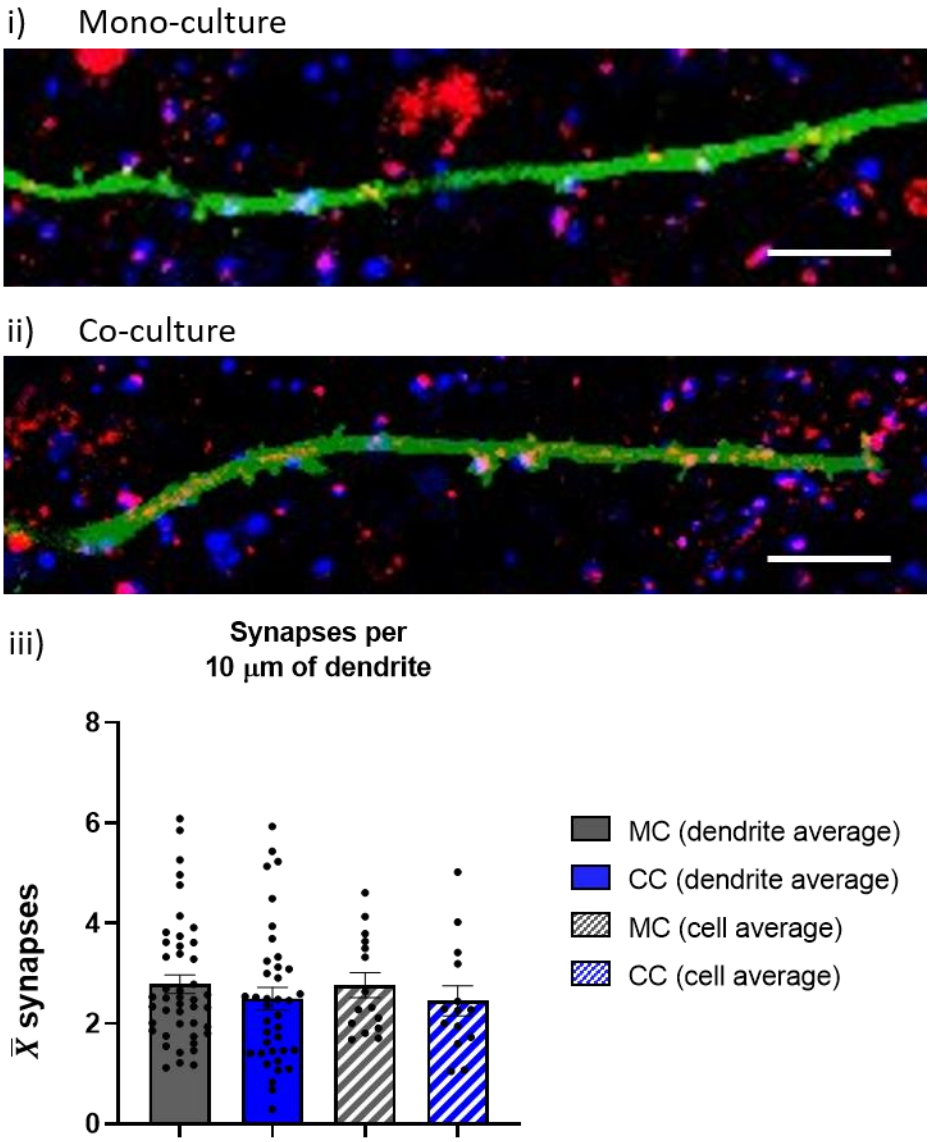


Figure 5.5: Co-localised pre- and post-synaptic markers appear with the same frequency in mono- and co-cultured cortical neurons

Co-localised pre- and post-synaptic markers along GFP-positive dendrites were counted (3 dendrites per cell). i & ii) example of MC and CC dendrites. Co-localised puncta staining is seen along both MC and CC dendrites. iii) There was no difference in the average number of synapses over a given length of dendrite between MC and CC cortical neurons. Astrocytes do not increase the number of structural synapses in cortical neurons (p = 0.1, LME ANOVA, df = 25, 3 independent cultures). Scale = 5 μm; pre-synapse (Synapsin-1) = blue channel, post-synapse (Homer-1) = red channel, GFP = green channel. Pink-purple = pre- & post-synapse overlap, pale yellow-pink = synapse-GFP overlap.

5.6 Lack of astrocytic influence on synaptogenesis not due to an inefficiency of our cortical astrocytes; synaptogenesis can be induced in RGCs

Substantial evidence has shown that astrocytes are needed for excitatory synapse formation in purified RGC cultures (Ullian et al., 2001, Nägler et al., 2001, Pfrieger and Barres, 1997). It has been largely suggested that this function of astrocytes is conserved throughout different brain regions, although there is little direct evidence that astrocytes are required for excitatory synaptogenesis in cortical cells.

My results showing that astrocytes have no effect on excitatory synapse formation therefore raise several questions. Firstly, is it a deficiency of my astrocyte preparation that lends them unable to induce synapse formation? This seems unlikely, given that I do see robust synaptic activity in my MC preparations, along with plentiful co-localised puncta. Secondly, is the lack of effect due to a difference in media, that is, is there some factor in my media that is supporting synaptogenesis, masking the role of astrocytes?

To address these questions, I established purified retinal ganglion cell culture preparations. As there is ample evidence to show astrocytes have a significant effect on RGCs, this allowed me to check that my astrocytes were able to induce synaptogenesis in these cells and confirm it wasn't the mouse astrocytes that were the problem. This also allowed me to check that it wasn't my media inducing synaptogenesis, masking an astrocytic effect.

I prepared rat RGCs from P5 pups, and plated the isolated cells directly onto coverslips, or else onto a bed of standard mouse astrocytes. Importantly, I fed the RGCs with my standard 1% NBA growth media – including rat serum and B27 (with the addition of BDNF, CNTF and forskolin that is required for RGC survival). I maintained the cells until DIV12 before recording.

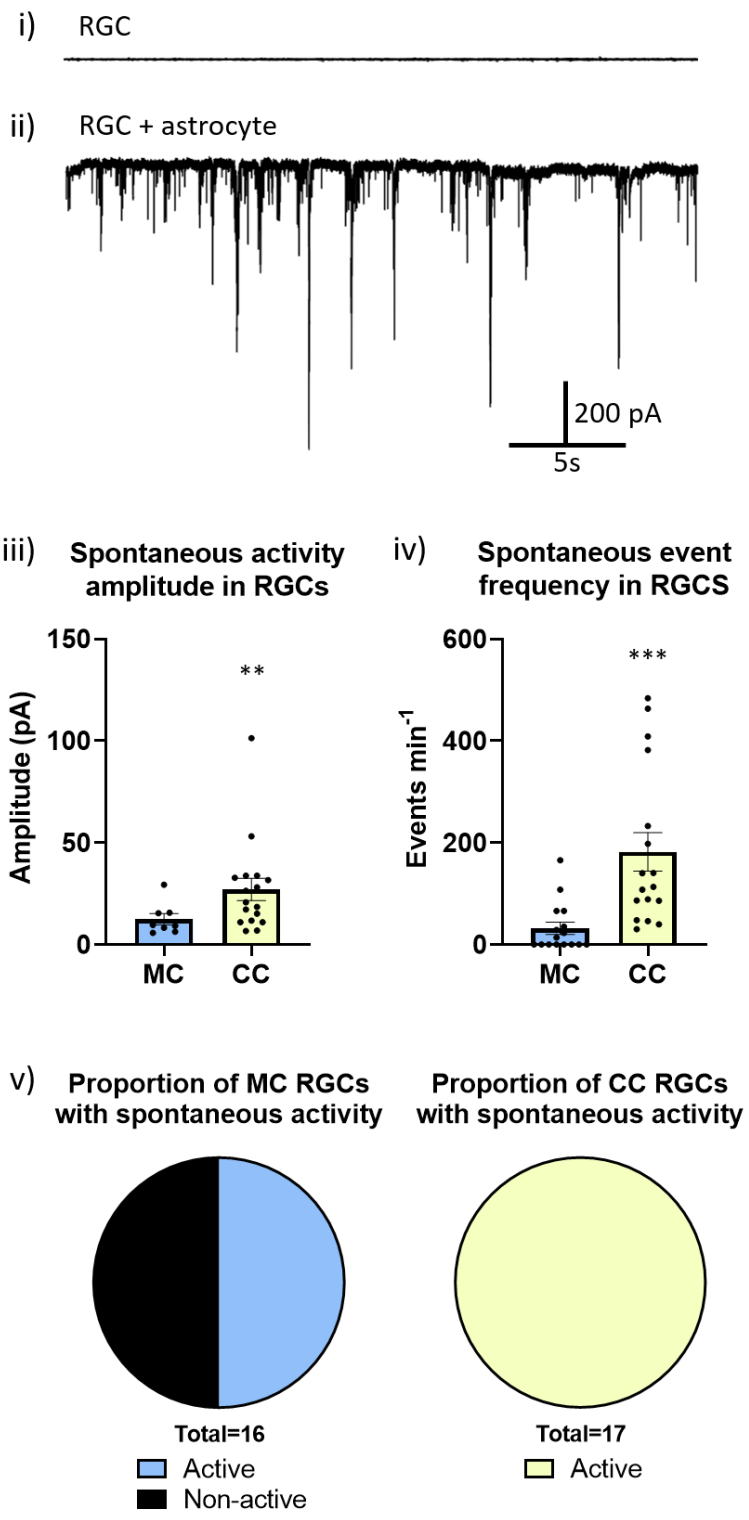


Figure 5.6: Spontaneous activity in retinal ganglion cells cultured alone or with mouse astrocytes

Retinal ganglion cells were recorded at DIV12. Compared to RGCs grown alone (i) there is a large induction of spontaneous activity in RGCs cultured on a bed of mouse astrocytes (ii). This increase is seen both in the average amplitude of events ($p = 0.002$, LME ANOVA, df = 28) and in the frequency of events ($p < 0.001$). v) Co-culture with astrocytes increased the proportion of cells that displayed spontaneous activity. From n = 4 independent cultures.

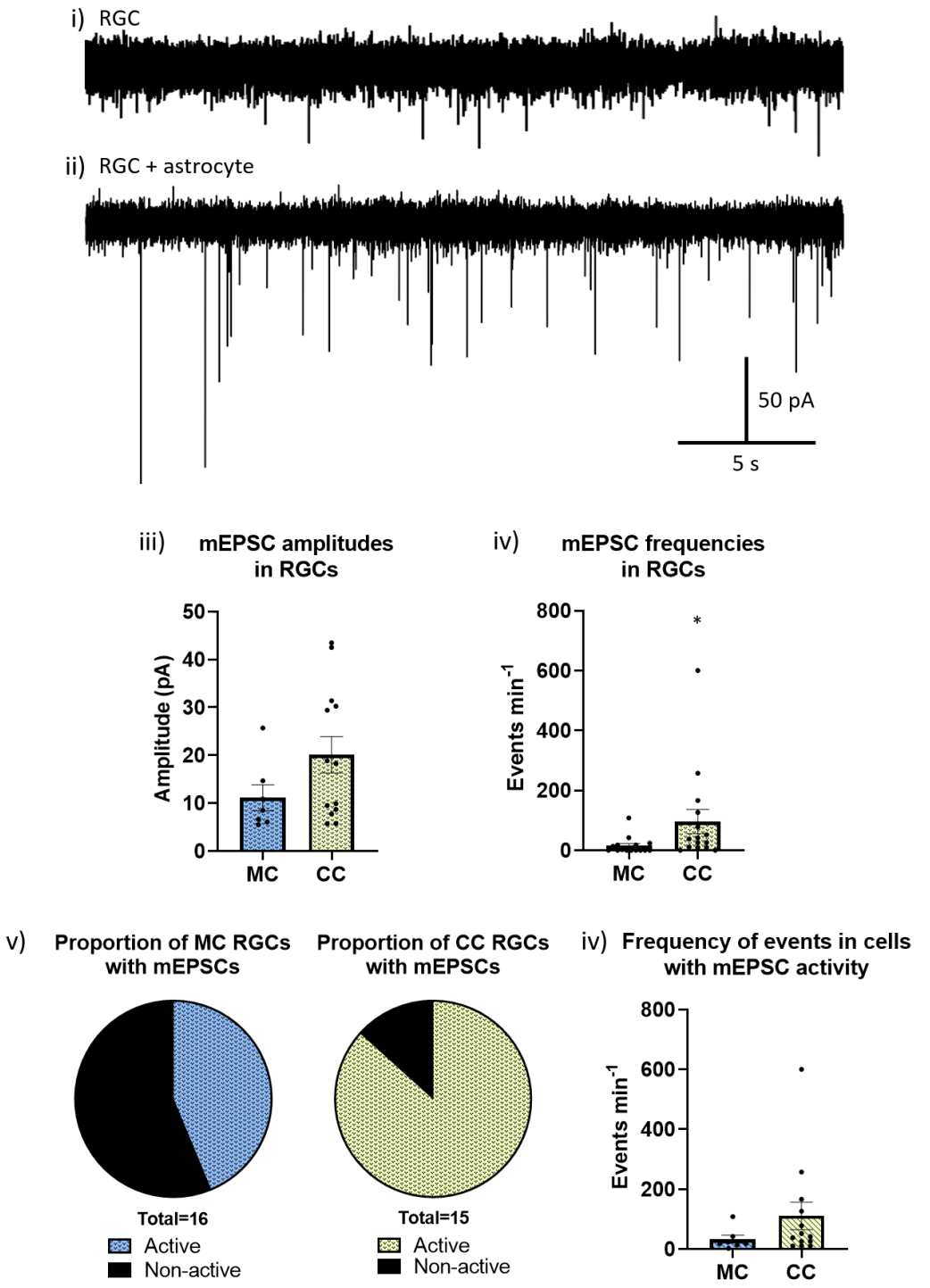


Figure 5.7: Astrocytes increase the proportion of retinal ganglion cells with synaptic activity and the frequency of mEPSC events

i) & ii) Example mEPSC recordings of RGCs. Cells were voltage-clamped at -70 mV and recorded in the presence of 300 nM TTX and 50 µM PTX. iii-iv) The addition of astrocytes increased the frequency ($p = 0.05$, LME ANOVA, df = 26) of mEPSC events in RGCs, but not the amplitude of events. v) Astrocytes increased the proportion of RGCs that were observed to have mEPSCs. iv) Of the RGC cells that displayed mEPSCs, the frequency was insignificantly larger in RGCs co-cultured with astrocytes ($p = 0.2$). From 4 independent cultures.

To begin with I looked at the spontaneous activity of MC and CC RGCs. There was very little spontaneous activity in RGCs grown alone (*Fig 5.6 i*) compared to RGCs grown with astrocytes (*Fig 5.6 ii*). This was reflected by a significant increase in both the amplitude (*Fig 5.6 iii*: mean = 12.4 ± 2.8 and 27.1 ± 5.5 pA, n = 16 and 17, for MC and CC, respectively) and the frequency of spontaneous events (*Fig 5.6 iv*: mean = 31.7 ± 12.0 and 182.1 ± 37.6 events min^{-1}, MC and CC, respectively). Most impressively, only half of the MC RGCs displayed any form of spontaneous activity, whereas spontaneous activity was seen in all RGCs grown on astrocytes (*Fig 5.6 v*).

I next looked at the mEPSC activity in RGCs. Unlike cortical cells, RGCs cultured with astrocytes had significantly more mEPSC events (*Fig 5.7 iv*: mean = 15.1 ± 7.4 and 96.1 ± 40.5 events min^{-1}, n = 16 and 15, MC and CC, respectively). Similar to spontaneous events, nearly all RGCs cultured with astrocytes had mEPSC events, whereas mEPSCs were seen in under half of the MC RGCs (*Fig 5.7 iv*).

From this I see that my astrocytes are able to replicate the results found in earlier studies, that is they increase both the spontaneous and mEPSC activity of RGCs. Importantly, astrocytes increase the proportion of RGCs that display both spontaneous and mEPSC activity, showing that they do indeed induce synaptogenesis in RGCs. Furthermore, I have ruled out that it is some factor in my media masking the synaptogenic effect of astrocytes in my cortical cells.

5.7 Homeostatic increases in synaptic strength after activity deprivation are only seen in co-culture

It has been well documented that 24-48 hours of activity deprivation results in a homeostatic increase in synaptic strength of neurons (Turrigiano et al., 1998). I showed in *Chapter 4.7* that the excitability of cortical neurons undergoes homeostatic regulation in response to activity deprivation, but that this only occurs in neurons grown with astrocytes. In this section I investigate whether the synaptic strength, as measured by mEPSC frequency and amplitude, of mono-cultured neurons is able to undergo this documented form of homeostatic plasticity, or whether co-culture with astrocytes is required.

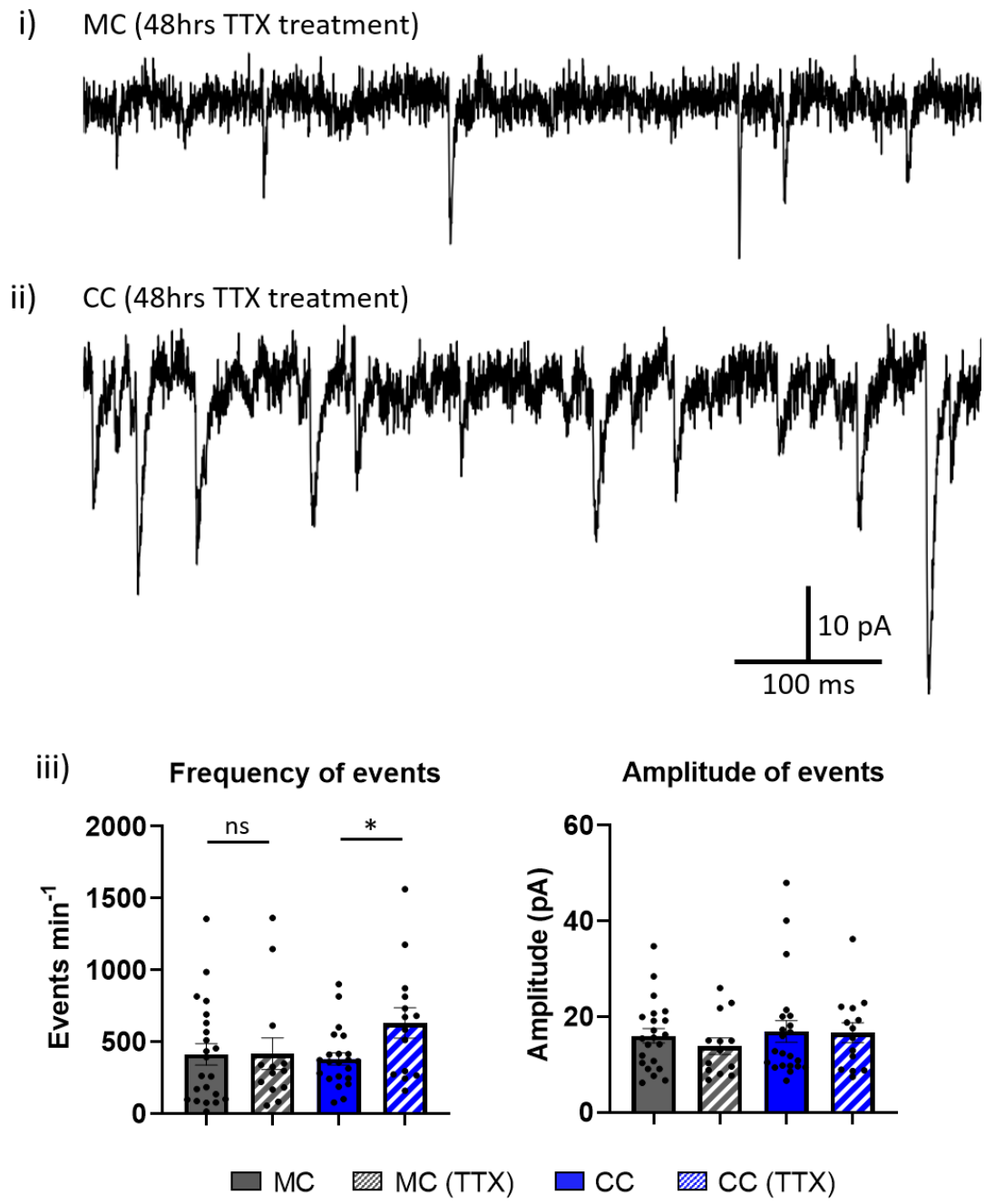


Figure 5.8: Blocking activity in DIV15 co-cultured neurons increases mEPSC frequency, but has no effect on mono-cultured neurons

The homeostatic increases in synaptic strength known to occur with activity deprivation are only observed in cortical neurons cultured with astrocytes. Mature cortical cultures had their activity blocked with 48 hrs of TTX treatment (300 nM) from DIV13. i) & ii) show example recordings from DIV15 MC and CC neurons, respectively, that had undergone activity blockade for 48 hrs. All recordings were done in the presence of 300 nM TTX and 50 µM PTX. iii) A homeostatic increase in event frequency due to activity deprivation is seen in CC neurons (p = 0.04, LME ANOVA, df = 56). There was no effect of activity deprivation on the amplitude of mEPSC events at DIV15 in either MC or CC cells. Recordings from 4 independent cultures.

I recorded mEPSCs from DIV15 MC and CC neurons which I had previously applied TTX to for 48 hours in order to block activity. There was no effect of activity deprivation on either the frequency or amplitude of mEPSC events on MC cells treated with TTX for 48 hours compared to control MC cells (*Fig 5.8, iii*: mean amplitude = 16.0 ± 1.5 and 13.8 ± 1.7 pA, for MC and MC +TTX, respectively, mean frequency = 412.7 ± 75.0 and 417.2 ± 110.7 events min^{-1}, for MC and MC +TTX, respectively; n = 23 & 13 cells, 4 independent cultures).

On the other hand, in co-cultured neurons there was a significant increase in the frequency of mEPSC events after activity deprivation (*Fig 5.8 iii*: mean = 381.3 ± 43.4 and 631.7 ± 105.1 events min^{-1}, n = 22 and 14, CC and CC +TTX, respectively). As with MC cells, there was no effect on the amplitude of mEPSC following activity deprivation (mean = 16.9 ± 2.3 and 16.6 ± 2.1 pA, for CC and CC +TTX, respectively). This shows that astrocytes are required for the activity-dependent homeostatic regulation of synaptic strength, as well as excitability.

5.8 Homeostatic regulation of neuronal activity only seen in co-cultured neurons

I showed in *Chapter 5.3* that there was significantly greater activity in CC neurons compared to MC neurons at DIV8. At this time point there was also significantly greater excitability in CC, which I hypothesised to be driving the observed increase in activity. By DIV15 I saw little difference in excitability between MC and CC neurons, so I next investigated whether there was a corresponding reduction in the difference of spontaneous network activity between DIV15 MC and CC cells.

I found that by DIV15 there was only a slight difference in spontaneous activity between MC and CC cortical neurons, with CC neurons seen to have just over twice the charge crossing the membrane as MC neurons (*Fig 5.9 v:* mean charge min^{-1} = 50.0 ± 14.9 and 116.8 ± 26 nC, n = 23 and 22, for MC and CC respectively, n = 7 independent cultures). By comparison, at DIV8 there was an ~10-fold increase in charge transfer in cortical neurons by co-culture with astrocytes, from ~25 nC min^{-1} in MC to ~235 nC min^{-1} in CC (*Fig 5.2*). This decrease in spontaneous activity with development in CC neurons is consistent with excitability playing a role in driving network activity.

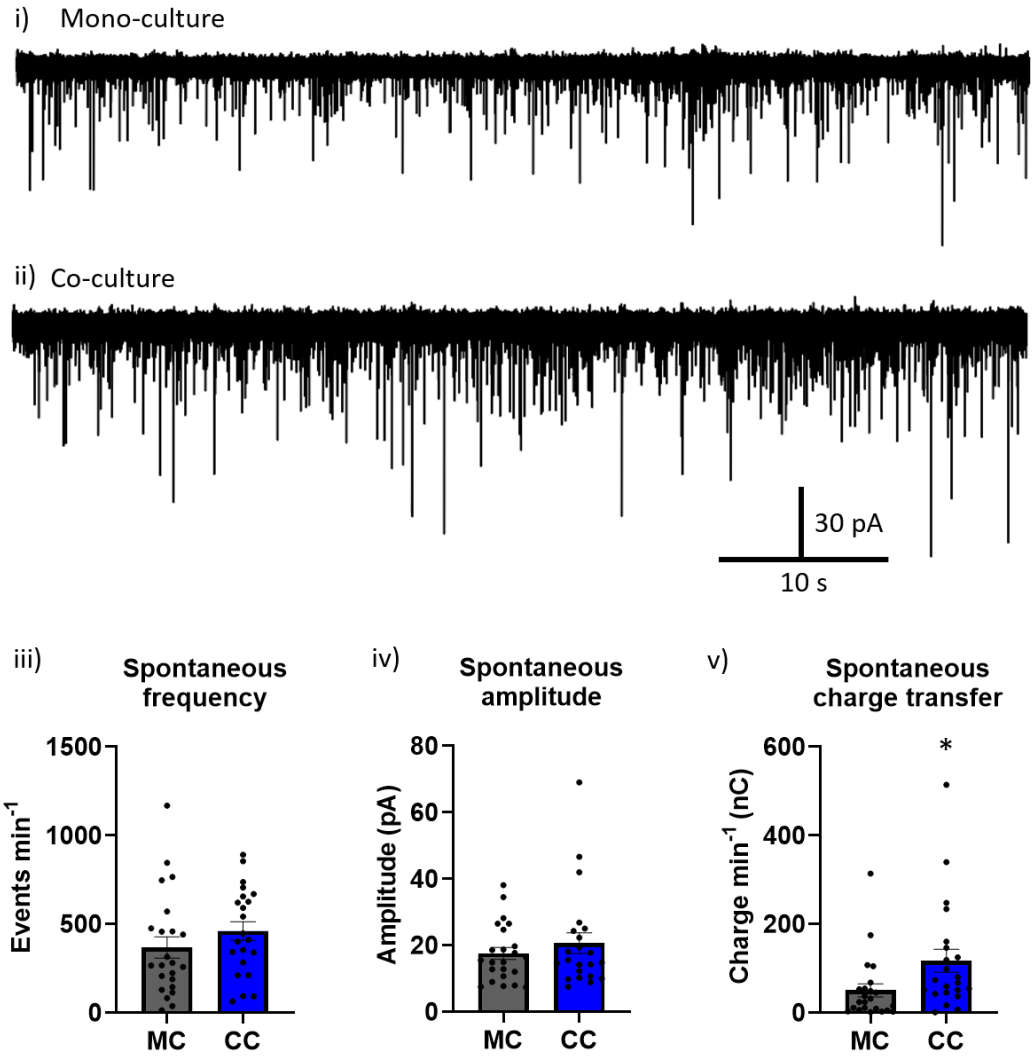


Figure 5.9: There is still a difference in spontaneous activity between MC and CC neurons by DIV15

Example traces of spontaneous activity recorded in DIV15 i) MC and ii) CC cortical neurons. iii & iv) There was no significant difference between the frequency and amplitude of spontaneous events between MC and CC neurons at DIV15. v) By DIV15 the difference in charge transfer of spontaneous events between MC and CC had diminished but was still significantly greater in CC neurons (p = 0.01, LME ANOVA, df = 37). From n = 7 independent cultures.

As I have shown both excitability and synaptic strength are upregulated in CC neurons following activity deprivation at DIV15, I then investigated the spontaneous network activity in response to activity deprivation at this time point, predicting that there would be an increase in activity specifically in CC neurons. As in the previous section, I applied TTX from DIV13 to block action potential driven activity in MC and CC neurons and recorded the spontaneous activity on DIV15 following TTX washout.

In activity-deprived MC neurons (MC +TTX) I saw very few large "network" events (*Fig 5.10 i*) following TTX removal, similar to untreated DIV8 and DIV15 MC neurons. This is marked by an insignificant difference in spontaneous activity following activity deprivation compared to untreated control MC neurons (*Fig 5.10 iii*: mean charge min^{-1} = 90.7 ± 64.6 nC, n = 13 for MC +TTX from 4 independent cultures).

Following TTX removal from CC neurons I saw robust regular network events (*Fig 5.10 ii*), in striking contrast to MC neurons. This was measured by a significant increase in the charge between activity-deprived MC and CC neurons (*Fig 5.10 iii*: mean charge min^{-1} = 90.7 ± 64.6 vs 1,710.6 ± 304.9 nC, n = 13 and 14, for MC +TTX and CC +TTX, respectively, 4 independent cultures), as well as a nearly 15-fold significant increase above the activity I saw in untreated control CC neurons.

By DIV15 there was little difference in spontaneous activity between MC and CC neurons but following 48 hours of activity deprivation there was a dramatic increase in network activity in cortical neurons grown with astrocytes. This activity-dependent homeostatic plasticity of network activity is not observed in the absence of astrocytes and is more pronounced than the homeostatic effects seen on either excitability or synaptic strength alone in CC neurons.

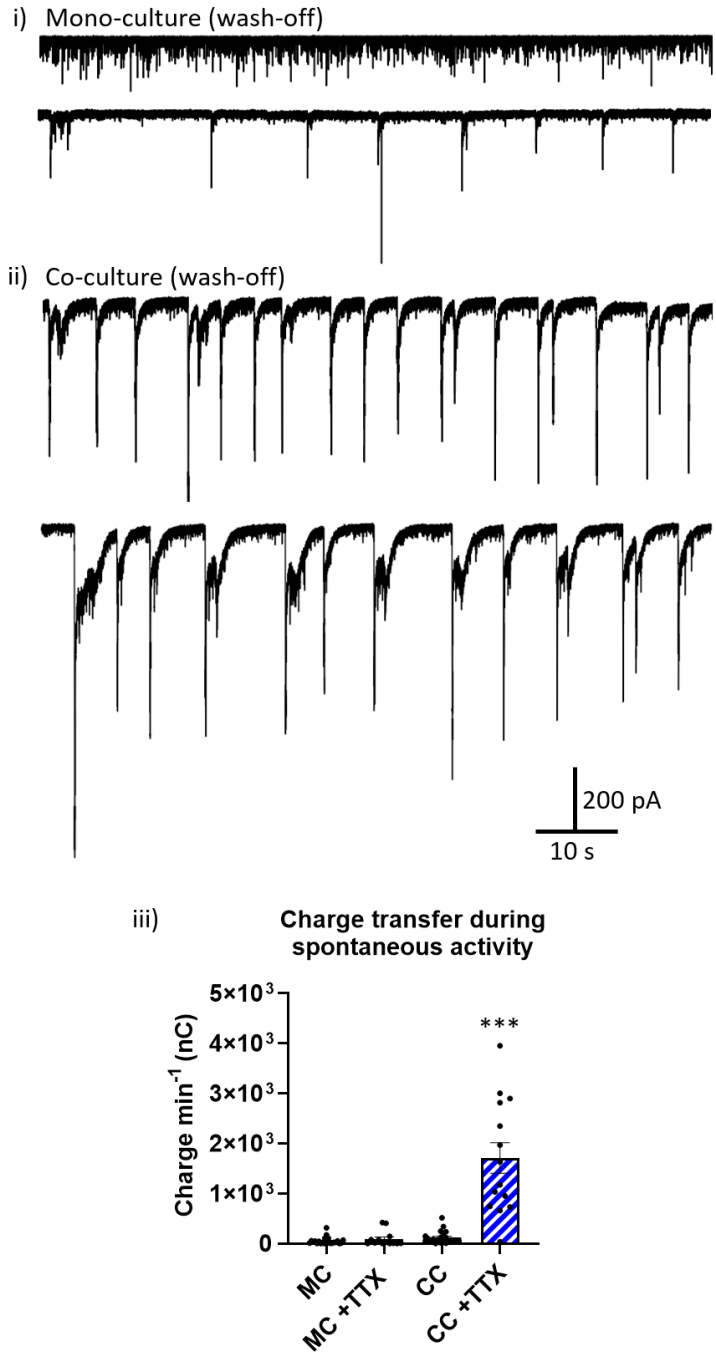


Figure 5.10: Removing TTX after 48hrs of activity inhibition induces robust neuronal network activity in co-culture, with limited effect in the absence of astrocytes

Removing TTX from DIV15 neurons after 48 hrs induces robust network firing events, with currents likely generated by voltage-gated Ca^{2+} and Na^{+} channels, in CC neurons. i) Example recordings of spontaneous activity in two DIV15 MC neurons following removal of TTX. Often there were no large events, as seen in the first trace. ii) Spontaneous activity of two DIV15 CC neurons following removal of TTX. Unlike MC cells, large burst events were seen in all CC neurons following TTX removal. iii) There is a significant increase in the network activity, as measured by charge transfer, in CC neurons following activity deprivation (CC +TTX) compared to both activity-deprived MC neurons (MC +TTX) and to untreated CC neurons ($p < 0.001$ compared to MC, MC +TTX and CC conditions, LME ANOVA, df = 55). There was no difference in charge between DIV15 MC +TTX and either control MC or CC conditions.

5.9 Discussion

5.9.1 Summary of findings

In this chapter I have shown that, consistent with an astrocyte-mediated increase in cortical neuronal excitability, there is an increase in spontaneous activity of cortical neurons grown in the presence of astrocytes. I further demonstrated this link with excitability, as by DIV15 the difference in activity between MC and CC neurons has largely disappeared, as I had observed the difference in excitability to do in *Chapter 4*. Unexpectedly, I found that under baseline activity conditions there was no increase in excitatory synapse numbers in cortical neurons cultured with astrocytes. In fact, I demonstrated that cortical neurons can develop extensive excitatory synapses in the absence of astrocytes. This is contrary to documented findings in RGCs, which I was able to replicate using my cortical astrocyte preparation, suggesting synaptogenesis in RGCs occurs via a different underlying mechanism to cortical neurons. However, I do find that astrocytes are able to regulate cortical excitatory synapses in an activity-dependent manner, showing that following activity deprivation there is a significant increase in excitatory synaptic strength only in cortical neurons grown with astrocytes. As I have shown astrocytes to be involved in the homeostatic regulation of excitability in *Chapter 4*, and now in this chapter to be involved in homeostatic regulation of synaptic strength, I investigated the total effect of astrocytes on the homeostatic plasticity of neuronal activity. I showed that astrocytes control neuronal activity levels overall, mediating a robust increase in cortical neuronal activity in response to activity deprivation.

5.9.2 Astrocytes increase the activity of neurons in a way consistent with an enhancement of excitability due to downregulation of K_{IR}

In this chapter I began by showing that increasing K_{IR} expression can decrease spontaneous firing of cortical neurons, demonstrating that, in principle, astrocytic control of K_{IR} expression could affect neuronal activity. As hypothesised from the increase in excitability seen in DIV8 neurons grown with astrocytes in *Chapter 4*, I showed that at DIV8 there were significantly higher spontaneous activity levels in CC cortical neurons. Furthermore, by DIV15 this difference in spontaneous activity had largely decreased. I predicted this to occur if the increase in activity seen at DIV8 was mediated by a decrease in K_{IR} expression and increase in excitability, as I had shown in

Chapter 4 that by DIV15 K_{IR} expression had increased in CC and there was no longer a difference in excitability between MC and CC cortical neurons.

The increase in activity due to astrocytes that I saw in this chapter supports many previous findings in RGCs. The consistent finding across the literature investigating the effects of astrocytes on RGCs has been that either astrocytes or astrocyte conditioned media is able to increase activity in RGCs (Allen et al., 2012, Ullian et al., 2001, Pfrieger and Barres, 1997, Christopherson et al., 2005, Mauch et al., 2001). As I mentioned in *Chapter 4*, none of these previous studies investigated whether astrocytes caused an increase in the excitability of RGCs that might explain this increase in spontaneous activity – they only investigated whether there was an increase in excitatory synapses and synaptic activity. I have shown in this chapter that for cortical neurons the increase in spontaneous activity is consistent with the changes of excitability seen in neurons due to astrocytes, suggesting that alterations in RGC excitability may also be contributing to the previously reported effect of astrocytes on activity in these cells.

5.9.3 Cortical neurons do not need astrocytes for the formation of excitatory synapses under baseline conditions

Although I did not find any effect of astrocytes on the gene expression of excitatory synapse-associated components in cortical neurons in *Chapter 4*, I hypothesised that the increase in activity might play a role in the previously reported astrocytic promotion of excitatory synaptogenesis, for example by increasing the intrinsically expressed pre- and post-synaptic puncta components' membrane formation and co-localisation. To see if the astrocyte-induced increase in activity was involved in astrocyte-mediated synaptogenesis, I started off by recording mEPSC events in DIV8 MC and CC neurons to confirm that there was a difference in excitatory synapses due to astrocytes. To my great surprise I found no difference in mEPSC events between MC and CC cortical neurons. Given the wealth of literature to the contrary (Mauch et al., 2001, Nägler et al., 2001, Pfrieger and Barres, 1997, Allen et al., 2012, Blanco-Suarez et al., 2018, Farhy-Tselnicker et al., 2017, Kucukdereli et al., 2011, Eroglu et al., 2009, Singh et al., 2016, Stogsdill et al., 2017, Risher et al., 2018) I began to consider why I could not replicate this finding, and what I must be doing wrong.

The first thing I considered was the age of the cells. I was recording at DIV8, and at this point the cultured rat cortical neurons are still quite immature, being before the point of spinogenesis. Work

by the lab of Eroglu and colleagues on the effect of hevin and excitatory synapse formation saw that this factor was required to prevent multiple synaptic inputs coalescing on a single spine (Risher et al., 2014). I reasoned then that it was possible the synapse promoting effect of astrocytes was happening later in development during spine formation. Therefore, I repeated my experiment at DIV15, by which point my cultured rat neurons had developed spines, to see if there was an excitatory synapse promoting effect of astrocytes apparent by this later stage. But, yet again, I failed to see a difference in mEPSCs between MC and CC neurons.

Up until now I had been using electrophysiological read-outs as a proxy for excitatory synapse number. To confirm the lack of difference I was observing in mEPSCs was representative of underlying excitatory synapse numbers I turned to immunohistochemistry to visual co-localised excitatory puncta in DIV15 neurons. Even so, I still saw no difference in co-localised pre- and post-synaptic excitatory puncta. As such, all evidence appears to suggest that astrocytes are not required for excitatory synaptogenesis in cortical neurons (and by extension that my hypothesis that the astrocyte-induced increase in activity may drive excitatory synapse formation was wrong).

5.9.4 Astrocyte induced synaptogenesis is still observed in RGCs: RGC synaptogenesis is separate to cortical synaptogenesis

Although my results above appear to suggest astrocytes are not involved in cortical synaptogenesis, it remained possible that either my astrocytes were somehow deficient in their ability to induce synaptogenesis, or else something in my media, for example the serum, was masking the astrocyte mediated effect on synaptogenesis. The most convincing demonstrations of astrocyte involvement in synaptogenesis have all been conducted using purified RGC cultures (Ullian et al., 2001, Pfrieger and Barres, 1997, Christopherson et al., 2005, Allen et al., 2012, Kucukdereli et al., 2011). Therefore, to rule out either of these possibilities I turned to RGCs to find out if I could replicate the well-established findings of astrocyte induced synaptogenesis in these cells using my astrocytes and media.

Consistent with previous reports I found that there was a significant increase both in spontaneous activity and mEPSC frequency in RGCs grown in the presence of my cortical astrocytes, that were fed with the same media as my cortical preparation. Also similar to earlier studies, I found that my astrocytes significantly increased the proportion of RGCs that displayed spontaneous activity,

finding spontaneous activity in 50% of MC RGCs and in 100% of CC RGCs; this is comparable to the findings of Pfrieger and Barres (1997) who observed spontaneous activity in 63% of their MC RGCs and 100% of RGCs grown with glia.

Therefore, the lack of effect of astrocytes on excitatory synapse numbers in my cortical neurons was not due to problems with my astrocytes, nor due to occluding factors in my media. Instead, the astrocyte-induced excitatory synaptogenesis reported in RGCs appears to be separate to the induction of excitatory synapse formation in cortical neurons. This should not be too surprising: as discussed in *Chapter 1.8.2.5*, RGCs may not be the best model for the neuron-astrocyte involvement in synapse development of the wider CNS. Importantly, RGCs do not normally form synapses with each other *in vivo*, so the lack of synapse formation observed in cultures consisting solely of purified RGCs may simply be due to an absence of their physiological synaptic partners, and not necessarily due to a lack of astrocytic factors. Supporting this, Pfrieger and Barres (1997) even reported that purified neurons from one of the RGC axon target areas, the superior colliculus, had the same pro-activity and synapse-inducing effects as astrocytes, which suggests that even for RGCs astrocytes may not necessarily be needed for excitatory synaptogenesis. My findings in this chapter that cortical neurons grown alone can develop synapses to the same extent as their counterparts grown on astrocytes, contrary to RGCs, highlights the potential shortcomings of using RGCs as a general model for neurons beyond the retina. Given that it is now possible to grow neurons from other CNS regions in the absence of glia it would be beneficial for future work attempting to draw conclusions about astrocyte-neuron interactions in other brain regions to utilise culture models using neurons from these regions instead of RGCs.

5.9.5 Astrocytes *do* regulate excitatory cortical synapses in an activity-dependent manner

Although I do not find that astrocytes are required for excitatory synapse formation under baseline conditions, I do see that they are required for homeostatic increases in synaptic strength following activity deprivation, and thus can mediate an increase in excitatory synapses. Given that I had found that astrocytes were required for the homeostatic plasticity of neuronal excitability in *Chapter 4*, and that previous work had also demonstrated their involvement in homeostatic plasticity of synaptic strength in hippocampal neurons (Stellwagen and Malenka, 2006), in this chapter I investigated their involvement in this form of homeostatic plasticity in cortical neurons. I found

that following 48 hours of activity deprivation of mature cortical neurons there was a robust increase in mEPSC frequency only in neurons grown in the presence of astrocytes, with no such homoeostatic increase in synaptic strength in MC cortical neurons. My finding suggests that astrocytes are also required for homeostatic plasticity of synaptic strength in cortical neurons, along with their documented role in this form of plasticity in hippocampal neurons. While I have shown that the astrocytes' homeostatic control of excitability is presumably mediated in part by changes in K_{IR} expression, I have not investigated whether this is true for the regulation of synaptic strengthening, or if this is mediated by a different astrocytic signalling pathway, for example the previously proposed astrocytic factor TNF-α involved in hippocampal plasticity (Stellwagen and Malenka, 2006).

My findings in this chapter show that astrocytes can promote excitatory synapse formation in an activity dependent manner. Thus, it is possible that some of the astrocytic factors found to be involved in RGC synapse formation are instead physiologically involved in the homeostatic regulation of synaptic strength in cortical cells.

5.9.6 The combined ability to regulate excitability and synaptic strength gives astrocytes significant control over the activity-dependent homeostasis of neurons

The spontaneous activity of neurons is mediated both by their excitability and their synaptic strength. As I saw in *Chapter 4* that the difference in neuronal excitability between MC and CC neurons had largely disappeared by DIV15, and as I saw no difference in synaptic strength between MC and CC cortical neurons, I hypothesised that the enhanced spontaneous activity in CC neurons I saw at DIV8 would have largely disappeared by DIV15. I demonstrated that this was indeed the case, with the enhancement of synaptic activity in CC neurons compared to MC neurons greatly diminished by DIV15. This reduction in spontaneous activity of CC neurons is quite clear to see when you compare the example traces of spontaneous activity from DIV8 to DIV15, with a disappearance of the large "action potential" events (*Fig 5.2 ii & Fig 5.9 ii*).

The forms of homeostatic plasticity that control excitability and synaptic strength that I have discussed in this and the previous chapter are both mechanisms with the ultimate purpose of regulating the activity of neurons in response to pathological activity levels. That is, in response to pathologically high levels of activity in the network they work together to bring activity down to

stable levels, whilst in response to an absence of activity they drive activity up (Stegen et al., 2011, Davis, 2013). As I have now demonstrated that astrocytes are required for the homeostatic increase of both excitability and synaptic strength in response to reduced activity, I predicted that this combined control would result in a robust increase in neuronal activity of CC neurons following activity deprivation. I found that this was the case: following 48 hours of activity deprivation there was a dramatic induction of neuronal network activity in neurons grown with astrocytes. Furthermore, in the absence of astrocytes there was no significant increase in activity, supporting my findings that neither excitability nor synaptic strength are upregulated following activity deprivation in the absence of astrocytes. My work here demonstrates that astrocytes are essential players in the homeostatic upregulation of cortical neuronal activity.

5.9.7 Limitations and future work

As was discussed in *Chapter 4,* a major limitation with this work is that it does not conclusively link astrocyte regulation of neuronal K_{IR} function to the increase in activity. Much of the future work I will do to determine this link has been outlined in the previous chapter, for example directly measuring the K_{IR} currents and determining the signalling pathway responsible for the reduction in cortical neuronal K_{IR} mRNA. Once I have found the pathway, I will investigate whether inhibiting it in CC neurons prevents the increase in neuronal activity, or whether activating the pathway in MC neurons boosts activity. In the meantime, I will investigate whether chronic partial inhibition of K_{IR}, for example with tertiapin Q and ML133, in MC neurons is able to boost activity in these cells.

Aside from this previously discussed gap, I have not yet investigated the effect of a stimulated increase in activity on the homeostatic responses of neurons. It is possible the baseline levels of activity in MC neurons were so low that activity deprivation had next to no effect, hence no homeostatic response was observed. Stimulating activity in MC and CC neurons, for example by application of the GABA receptor antagonist bicuculline and/or the voltage-activated K^+ channel blocker 4-Aminopyridine, to trigger homeostatic downregulation of activity would be one way to investigate this possibility further. Furthermore, this will also allow me to investigate whether astrocytes are involved in the reverse homeostatic downregulation of activity.

Additionally, as I have seen that activity deprivation of DIV15 CC neurons, but not MC neurons, leads to an increase in mEPSC events, I wish to see whether there is an associated increase in co-localised synaptic puncta in TTX-treated CC neurons. To do this I will repeat the immunohistochemistry experiments in DIV15 MC and CC neurons that have been treated with TTX and count the number of excitatory synapses present.

Lastly, all my work to date is in primary cortical culture preparations. Some of the criticism raised regarding the pitfalls of using purified RGCs as a model to study synapse formation and development could also be levelled at this model. For example, many of these cortical cells would physiologically receive input from areas beyond the cortex, such as the thalamus. Indeed, Eroglu and colleagues have demonstrated that the putatively astrocyte-secreted protein hevin appears to work to stabilise and increase the number of vGlut2-thalamocortical excitatory synapses – an effect my cortical preparation would miss (Singh et al., 2016, Risher et al., 2014). Once I have determined the signalling pathway mediating the decrease in K_{IR}/increase in excitability, the final part of my future work will look to generate a mouse model to study the relative importance of this astrocyte-to-neuron signalling pathway on excitability and network activity in an *in situ* and *in vivo* setting.

5.9.8 Conclusion

I have shown here that astrocytes are involved in controlling cortical neuronal activity. Intriguingly, I do not see that astrocytes are required for the induction of cortical neuronal excitatory synaptogenesis, questioning the suitability of RGCs as a model for neuron-astrocyte interactions in synapse formation. Despite this, I find that astrocytes can control excitatory synapses in cortical neurons in an activity-dependent manner, showing that astrocytes are required for the homeostatic upregulation of synaptic strength following activity deprivation. In combination with my findings that astrocytes are required for the homeostatic upregulation of excitability, I demonstrate that astrocytes exert a large control over the activity of cortical neurons and are required for activity-dependent homeostatic plasticity in these cells. This work adds to astrocytes known roles in other forms of plasticity, LTP and LTD, establishing these cells as having a prominent role in all forms of activity-dependent plasticity.

Chapter 6

Concluding remarks

Chapter 6 – Concluding remarks

Neurons and astrocytes share an intimate bidirectional relationship with each other within the CNS. However, traditionally it has been difficult to determine exactly how one cell type affects the other. Considering this, our lab has come up with a mixed species co-culture approach that allows us to ascertain how one type of cell regulates the gene expression of another. In my thesis I have used this approach to explore the extent of the reciprocal relationship between neurons and astrocytes. I first demonstrated that neurons play an important role in determining astrocyte function, before showing that astrocytes in turn have control over neuronal gene expression and activity.

Using our lab's mixed species co-culture setup, in the first half I showed how cortical neurons control the gene expression of the two astrocytic glutamate transporters, *Slc1a2* and *Slc1a3*, through the contact dependent Notch signalling pathway, and how this control leads to an induction of the astrocytic ability to clear glutamate. Astrocytic clearance of glutamate is a fundamentally important aspect of CNS function, with impairments in this clearance leading to epilepsy and excitotoxic cell death. It is intriguing to find that this ability, being one that so often characterises astrocytes and their function, is not innate to astrocytes and is instead controlled by neurons, highlighting the complex entwined relationship between these two cell types. My demonstration that Notch signalling from neurons is required to maintain the expression of these transporters suggests ways in which the breakdown in communication between these two cell types can have important consequences for the development of neurodegenerative disease. For example, a lack of Notch signalling will reduce astrocytic EAAT function, which could lead to excitotoxic neuronal death, which could cause a further reduction in contact and Notch signalling, a further reduction in glutamate clearance, and a potentiation of toxicity. An interesting avenue for future research would be to investigate the role impaired Notch signalling in *astrocytes* plays in the development of, for example, Alzheimer's disease – given familial cases of the disease often involve mutations in the γ-secretase complex.

In the second half of my thesis I presented our lab's RNA-seq results on the astrocytic regulation of neuronal gene expression, finding that astrocytes extensively control cortical neuronal gene expression. This is the first study revealing how astrocytes control neuronal gene expression and is

an important finding on its own. Among these astrocyte-controlled genes I discovered that the *Kcnj* family of genes was downregulated in cortical neurons by astrocytes. As these genes encode the K_{IR} channel subunits I hypothesised that this astrocytic control would have functional consequences for the neurons. I showed that this was the case, with astrocytes altering the membrane properties of neurons, increasing neuronal excitability, and in turn increasing neuronal activity. Finding the signalling mechanism behind this astrocyte control of *Kcnj* expression is the next key step for this work. A summary of two proposed candidate mechanisms and their pharmacological targets for my experiments going forward are depicted in *Figure 6.1*.

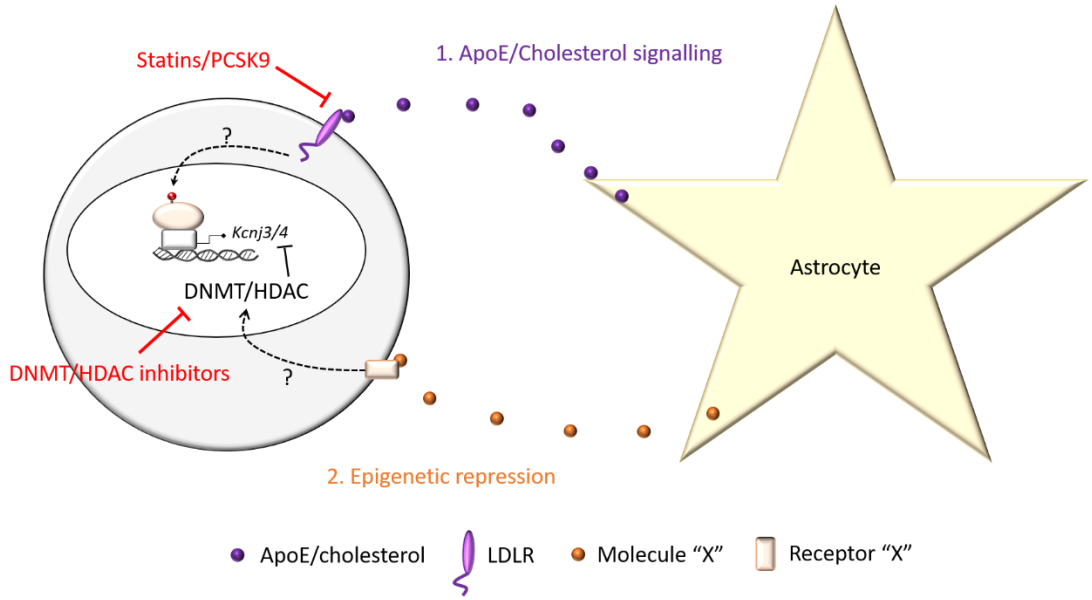


Figure 6.1: Proposed astrocyte signalling mechanisms leading to the repression of neuronal *Kcnj*

The primary targets for my work going forward are 1. the potential role of ApoE/cholesterol signalling leading to an inhibition of *Kcnj3/4* transcription and 2. whether the astrocytic downregulation of *Kcnj* is mediated by epigenetic repression of these genes in neurons. To investigate these possibilities, I will initially use pharmacological interventions to inhibit these two signalling pathways, for example by application of PCSK9 to reduce low density lipoprotein receptor (LDLR) expression, or DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors to prevent epigenetic repression.

Contrary to previous findings espousing the requirement of astrocytes for excitatory synaptogenesis I did not find genes associated with excitatory synapses to be regulated by astrocytes. In agreement with the RNA-seq dataset I found that there was no increase in excitatory synapse formation under baseline conditions in the presence of astrocytes. I proved that my astrocytes and media conditions

allowed for astrocyte induced synaptogenesis of RGCs, suggesting the results of previous studies using purified RGCs may not be generalisable to cells beyond that preparation.

Despite not seeing an increase in excitatory synapses in the presence of astrocytes, it was observed that the kinetics of the mEPSC events may be different between the two conditions. Specifically, the duration of the events appeared to be longer in the presence of astrocytes, as seen in *Figure 5.8.* This could indicate that there is a difference in the AMPA receptor subunit composition, with mono-culture neurons possessing more kinetically fast Ca^{2+}-permeable AMPA receptors compared to co-culture. This would be consistent with the observations of Allen and colleagues, who found that astrocytes promote the formation of Ca^{2+}-impermeable AMPA receptors in neurons (Blanco-Suarez et al., 2018). In the future it would be of interest to investigate this possibility further, for example by using Naspm, a Ca^{2+}-permeable AMPA receptor antagonist, to investigate whether there is a reduction in these receptors in the presence of astrocytes.

The major finding from the second half of my thesis was that astrocytes were required for activity-dependent regulation of neuronal excitability and synaptic strength. I showed that the regulation of these two mechanisms of activity-dependent homeostatic plasticity endowed astrocytes with a profound ability for the homeostatic regulation of neuronal activity. It has been established that astrocytes are closely involved in the forms of plasticity associated with memory, LTP and LTD, with one study also suggesting a role in the homeostatic plasticity of synaptic strength. The results from my thesis firmly cement astrocytes as players in the various aspects of homeostatic plasticity and suggests a major function of astrocytes in the CNS may be the regulation of neuronal plasticity.

In experiments going forward it will be desirable to investigate the relevance of these findings in a more physiologically intact setting. One interesting possibility would be to set up a dark rearing experiment to see if the expression of these K_{IR} channels decreases in the visual cortex in the absence of light and increases upon exposure to light. If this is the case, I would then investigate whether impairing the astrocyte-to-neuron signalling cascade could prevent these changes, which could demonstrate whether or not this physiological response in rodents requires astrocyte-to-neuron signalling.

Overall, in my thesis I have elucidated some of the reciprocal signalling mechanisms and associated functional control that neurons and astrocytes exert over each other. This work is just the beginning, providing a glimpse of the complex relationship that is becoming increasingly apparent between these two major cell types. Future work will then need to explore how the other glial cells, the oligodendrocytes, microglia and $NG2^+$ cells, in turn fit into this relationship with neurons and astrocytes.

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Appendix

Figure A1

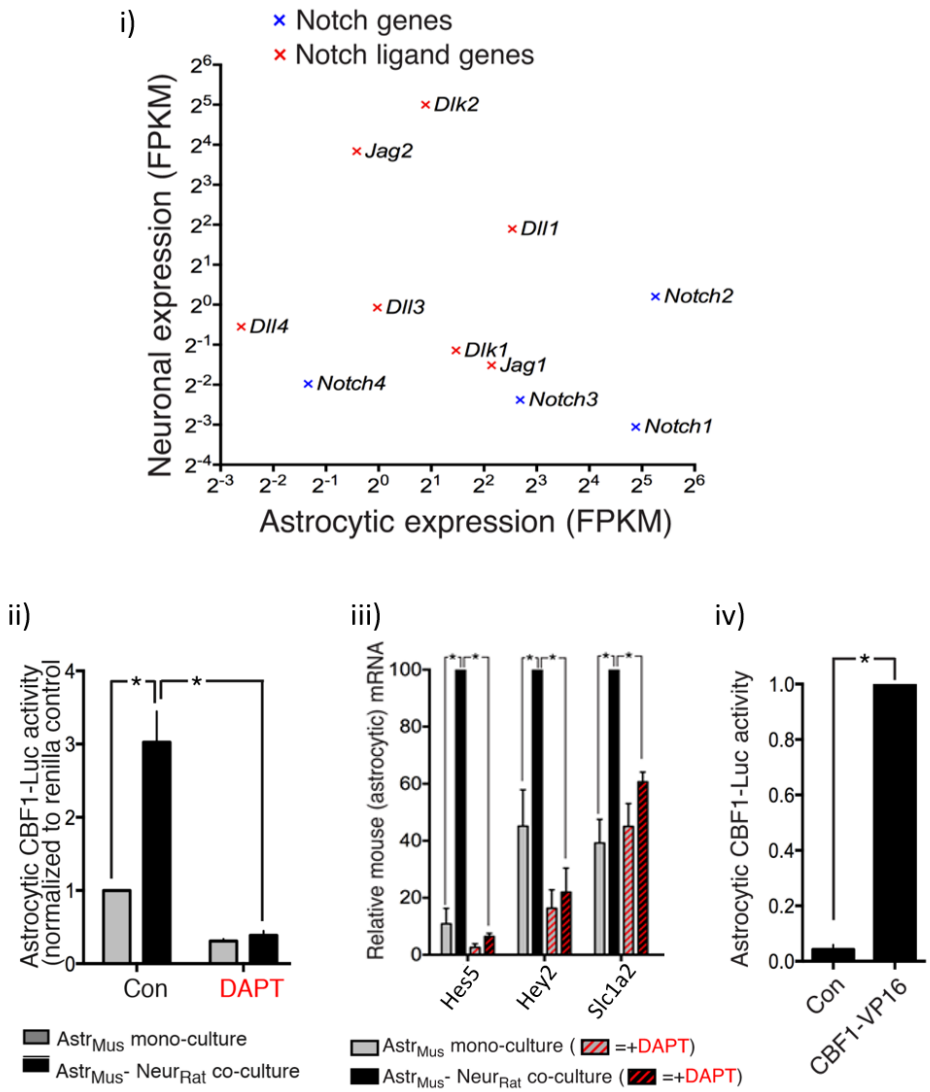


Figure A1: Involvement of the Notch signalling pathway between neurons and astrocytes

All figures are taken from Hasel et al., 2017, with data acquired by Dr. Philip Hasel. i) Relative expression of Notch ligands and Notch receptors in neurons vs. astrocytes. It can be seen that neurons are particularly enriched for the ligands, whilst astrocytes are enriched for the receptors. ii) Luciferase assay of the Notch effector, CBF1. There is greater Notch activity in astrocytes grown with neurons, and this activity can be blocked by inhibiting the cleavage of the NICD with DAPT. iii) Relative expression of the two Notch genes, *Hes5* and *Hey2*, along with the glutamate transporter *Slc1a2* in mouse astrocytes ± rat neurons. Neuronal inducement of gene expression is blocked for all three genes by application of the γ-secretase inhibitor DAPT to prevent Notch signalling. iv) Expression of the constitutively active Notch effector mutant causes an increase in Notch transcription in astrocytes as measured by a CBF1 luciferase assay.

Figure A2

Gene	Name	MC DIV8 FPKM	CC DIV8 FPKM	p_adj
TUBA1A	tubulin alpha 1a	2735.054	4296.278	5.48E-29
TUBB2B	tubulin beta 2B class iib	1845.898	3083.407	7.76E-36
TUBB3	tubulin beta 3 class iii	1406.655	2630.219	3.47E-48
TUBB5	tubulin beta class i	1098.184	1841.882	7.18E-40
TUBA1B	tubulin alpha 1B	388.6917	625.2883	8.48E-28
TUBB2A	tubulin beta 2A class iia	371.5229	561.1218	1.99E-28
TUBB4A	tubulin beta 4A class iva	249.9975	310.7856	4.29E-08
TUBB4B	tubulin beta 4B class ivb	159.1605	236.7573	5.26E-18
TUBA4A	tubulin alpha 4a	94.52494	114.7001	1.46E-05
TUBG1	tubulin gamma 1	30.01506	35.81264	4.02E-05
TUBG2	tubulin gamma 2	13.81496	14.52302	0.217793
TUBD1	tubulin delta 1	3.072431	3.635421	0.156286
TUBE1	tubulin epsilon 1	2.193553	2.289846	0.532555
TUBA8	tubulin alpha 8	1.832431	1.453416	0.444698
TUBB6	tubulin beta 6 class v	1.697915	0.620243	1.79E-06
TUBA1C	tubulin alpha 1c	0.066929	0.1808	0.187363
Total		**8402.382**	**13758.95**	

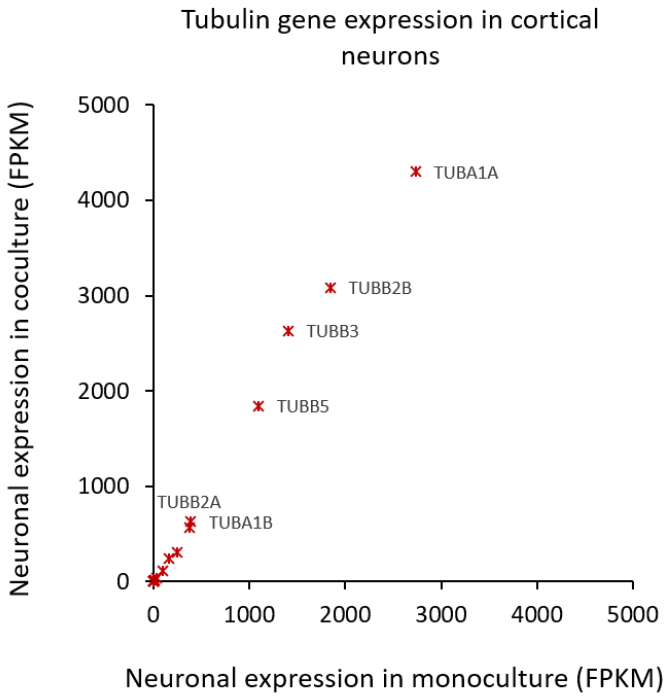


Figure A2: Tubulin expression in MC and CC cortical neurons (DIV8)

Tubulin gene expression is significantly greater for the majority of the tubulin gene family in rat cortical neurons grown in the presence of mouse astrocytes. *Tuba1a*, *Tubb2b, Tubb3* and *Tubb5* were some of the highest expressed genes in CC neurons, and amongst the most significantly upregulated by co-culture with astrocytes. Samples prepared by Dr Jing Qio and Dr. Philip Hasel, bioinformatic analyses undertaken by Dr Owen Dando and Dr Xin He.

Figure A3

Excitatory synapse genes							
Presynapse		**DIV8**			**DIV15**		
Gene	**Protein**	**MC (FPKM)**	**CC (FPKM)**	**P_adj**	**MC (FPKM)**	**CC (FPKM)**	**P_adj**
VAMP1	Vamp1	6.30	6.08	0.89	19.09	16.44	0.35
VAMP2	Vamp2	446.34	416.99	0.81	596.40	650.08	0.66
SNAP25	Snap-25	236.03	226.13	0.90	483.45	470.25	0.89
Stx1a	Syntaxin-1A	129.81	156.91	0.00	158.97	165.30	0.94
SYT1	Synaptotagmin	230.81	218.80	0.99	327.52	373.17	0.38
SYP	Synaptophysin	485.76	370.92	0.00	779.50	687.26	0.28
SYN1	Synapsin-1	194.39	175.43	0.32	279.92	284.65	0.99
Slc17a7	VGLUT1	148.44	162.09	0.00	304.73	342.30	0.55
Slc17a6	VGLUT2	23.42	16.62	0.00	47.22	30.59	0.63
Total expression (FPKM)		***1901.28***	***1749.96***		***2996.79***	***3020.04***	
post-synapse		**DIV8**			**DIV15**		
Gene	**Protein**	**MC (FPKM)**	**CC (FPKM)**	**P_adj**	**MC (FPKM)**	**CC (FPKM)**	**P_adj**
HOMER1	Homer-1	24.29	26.97	0.00	36.99	33.18	0.81
DLG4	PSD-95	179.38	229.79	0.00	268.15	264.48	0.95
NLGN1	Neuroligin-1	17.73	17.84	0.29	21.40	24.56	0.42
SYNGAP1	SynGAP	103.05	103.76	0.26	154.34	154.14	0.98
SHANK1	Shank-1	79.90	124.11	0.00	89.54	103.17	0.43
SHANK3	Shank-3	51.77	52.71	0.14	52.02	50.71	0.92
Total expression (FPKM)		***456.12***	***555.19***		***622.44***	***630.24***	
AMPA & NMDA receptor subunits		**DIV8**			**DIV15**		
Gene	**Protein**	**MC (FPKM)**	**CC (FPKM)**	**P_adj**	**MC (FPKM)**	**CC (FPKM)**	**P_adj**
GRIA1	GluA1	45.05	49.07	0.01	73.41	77.40	0.88
GRIA2	GluA2	61.51	53.67	0.13	92.49	90.89	0.94
GRIA3	GluA3	11.38	13.33	0.00	22.64	28.00	0.11
GRIA4	GluA4	27.35	28.03	0.15	38.20	40.86	0.81
GRIN1	GluN1	83.66	74.22	0.19	152.79	144.36	0.73
GRIN2a	GluN2a	1.91	1.37	0.03	4.30	4.46	0.97
GRIN2b	GluN2b	7.81	11.59	0.00	10.88	12.89	0.86
GRIN2d	GluN2d	9.24	9.62	0.14	9.42	8.92	0.90
GRIN3a	GluN3a	6.89	21.54	0.00	6.77	14.45	0.00
Total expression (FPKM)		***254.81***	***262.45***		***410.89***	***422.23***	

Figure A3: Gene expression of excitatory synapse associated genes for MC and CC cortical neurons at DIV8 and DIV15 as assessed from RNA-sequencing

Co-culture with astrocytes had very little effect on gene expression for excitatory synapse associated proteins, including pre-synaptic, post-synaptic and ionotropic glutamate receptor subunit proteins in cortical rat neurons. This was the case both early on in culture (DIV8), and also later on in development at DIV15, after the formation of spines had occurred. Samples prepared by Dr Jing Qiu and Dr Philip Hasel, bioinformatic analyses undertaken by Dr Owen Dando and Dr Xin He.

Figure A4

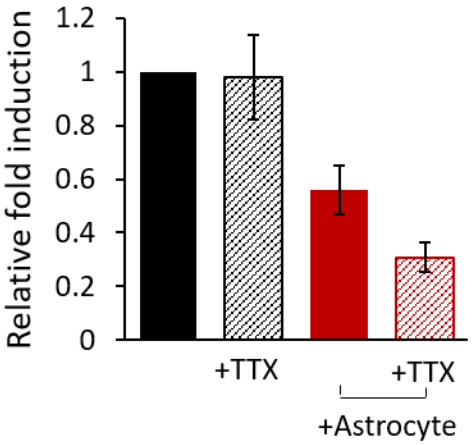
K_{IR} channels expressed in cortical neurons							
		DIV8			DIV15		
Gene	Protein	MC (FPKM)	CC (FPKM)	P_adj	MC (FPKM)	CC (FPKM)	P_adj
Kcnj2	Kir2.1	11.06	6.32	*3.76E-10*	5.53	4.95	*0.824*
Kcnj12	Kir2.2	1.70	3.02	*6.01E-06*	2.99	2.87	*0.955*
Kcnj4	Kir2.3	15.78	5.62	*1.84E-52*	38.86	25.48	*9.59E-05*
Kcnj14	Kir2.4	4.67	4.13	*0.557*	4.26	3.51	*0.573*
Kcnj3	Kir3.1	35.26	8.96	*5.1E-120*	30.81	21.62	*0.008*
Kcnj6	Kir3.2	4.54	3.44	*0.020*	4.91	4.81	*0.969*
Kcnj9	Kir3.3	7.02	4.29	*1.51E-06*	14.72	14.89	*0.986*
Kcnj11	Kir6.2	5.79	5.30	*0.78*	5.81	4.70	*0.367*
Total expression (FPKM)		***85.81***	***41.07***		***107.86***	***82.85***	

Figure A4: RNA-sequencing data of K_{IR} gene expression in cortical rat neurons is decreased in the presence of mouse astrocytes

Expression of K_{IR} channel genes in cortical neurons grown alone (MC) or in the presence of mouse astrocytes (CC) at DIV8 and DIV15. There is a pronounced reduction in expression at DIV8, which is somewhat reduced by DIV15. The largest effects are on the two most abundantly expressed members, *Kcnj3* (K_{IR}3.1) and *Kcnj4* (K_{IR}2.3). At DIV15 there is still a significant reduction of these two genes in the presence of astrocytes. Samples prepared by Dr Jing Qiu and Dr Philip Hasel, bioinformatic analyses undertaken by Dr Owen Dando and Dr Xin He.

Figure A5

i) Neuronal *Kcnj3* expression at DIV15



ii)

RNA-sequencing data of DIV15 cortical (rat) neuron K_{IR} expression

Gene	Protein	MC (FPKM)	MC+TTX (FPKM)	P_adj	CC (FPKM)	CC+TTX (FPKM)	P_adj
Kcnj2	Kir2.1	5.53	6.83	*0.342*	4.95	1.86	*1.41E-08*
Kcnj12	Kir2.2	2.99	2.90	*0.915*	2.87	3.65	*0.262*
Kcnj4	Kir2.3	38.86	26.93	*3.72E-09*	25.48	18.46	*3.96E-05*
Kcnj14	Kir2.4	4.23	3.75	*0.667*	3.51	4.00	*0.554*
Kcnj3	Kir3.1	30.81	24.73	*0.610*	21.62	13.46	*1.33E-05*
Kcnj6	Kir3.2	4.91	6.14	*0.265*	4.81	5.93	*0.113*
Kcnj9	Kir3.3	14.71	19.15	*0.382*	14.89	11.32	*0.004*
Kcnj11	Kir6.2	5.81	4.72	*0.784*	4.70	4.82	*0.996*
Total expression (FPKM)		***107.86***	***95.14***		***82.85***	***63.50***	

Figure A5: Activity dependent changes in K_{IR} expression in DIV15 cortical rat neurons

i) Relative expression of *Kcnj3* in cortical rat neurons ± mouse astrocytes with and without activity deprivation (by TTX application) at DIV15 as assessed by RT-qPCR (data courtesy of Dr Philip Hasel). Activity deprivation has little effect on *Kcnj3* expression in neurons in the absence of astrocytes. In the presence of astrocytes *Kcnj3* expression appears to be further reduced. ii) RNA-seq dataset of DIV15 MC and CC rat neurons (samples prepared by Dr Philip Hasel, bioinformatic analysis by Dr Owen Dando and Dr Xin He). Blocking activity for 48 hours with TTX treatment had little effect on the gene expression of K_{IR} channels in MC rat cortical neurons. In contrast, there was a greater repression of *Kcnj* genes in CC neurons following activity deprivation.

Figure A6

		Mouse neuron MC (-glia) gene expression (FPKM)			Mouse mixed culture (+glia) gene expression (FPKM)		
Gene	**Protein**	**Control**	**+Bic**	**+TTX**	**Control**	**+Bic**	**+TTX**
Kcnj2	Kir2.1	718.71	311.12	500.09	386.76	266.84	257.77
Kcnj12	Kir2.2	183.63	179.84	147.15	126.77	102.72	78.87
Kcnj4	Kir2.3	3409.00	2295.04	1438.83	2046.28	3212.12	1161.14
Kcnj14	Kir2.4	53.71	82.82	33.66	30.30	57.22	31.25
Kcnj3	Kir3.1	4338.85	3097.55	3793.54	3945.48	4290.78	2322.53
Kcnj6	Kir3.2	319.17	544.48	346.45	354.07	273.77	245.51
Kcnj9	Kir3.3	1232.30	679.19	1194.13	1105.98	598.08	980.68
Kcnj11	Kir6.2	203.02	159.83	288.19	206.16	128.70	188.32
Total expression (FPKM)		***10458.4***	***7349.87***	***7742.04***	***8201.81***	***8930.23***	***5266.07***

Figure A6: Activity regulation of K_{IR} channels in pure mouse neurons vs mixed mouse cells

RNA-seq data for *Kcnj* gene expression in mouse primary culture. Mouse neurons were either purified with AraC treatment from DIV0 (-glia), or else grown without to allow growth and proliferation of glial cells, including astrocytes (+glia). As with the rat data, there is a reduction in *Kcnj* expression in mouse samples containing glia. Increasing activity by application of bicuculline causes an increase in *Kcnj3* and *Kcnj4* only in the presence of astrocytes. Conversely, reducing activity with TTX treatment causes a robust decease in these channels' expression in the mixed sample, supporting our findings in rat preparations. NB: specific neuronal expression of *Kcnj* genes cannot be guaranteed in the mixed sample. Data obtained by Dr Nora Markus.

Figure A7

K_{IR} gene expression in DIV3 monoculture rat neurons ± ACM treatment

Gene	Protein	MC (FPKM)	MC+ACM (FPKM)	P_adj
Kcnj2	Kir2.1	14.05	11.20	*0.090352*
Kcnj12	Kir2.2	1.52	1.57	*0.992066*
Kcnj4	Kir2.3	4.75	2.53	*7.52E-06*
Kcnj14	Kir2.4	5.26	5.70	*0.88879*
Kcnj3	Kir3.1	18.86	8.66	*3.3E-14*
Kcnj6	Kir3.2	4.58	2.82	*0.000358*
Kcnj9	Kir3.3	4.00	3.08	*0.237306*
Kcnj11	Kir6.2	4.08	3.69	*0.764745*
Total expression (FPKM)		***57.11***	***39.25***	

Figure A7: Regulation of K_{IR} genes in DIV3 rat neurons by ACM treatment

RNA-seq data for *Kcnj* gene expression for MC rat neurons, grown in standard media or supplemented with ACM for three days. ACM treatment significantly reduced the expression of both *Kcnj3* and *Kcnj4*, replicating the effects of direct astrocyte co-culture on these genes. Samples prepared by Dr Philip Hasel, bioinformatics by Dr Owen Dando and Dr Xin He.

Figure A8 (1/4): Relative expression of ACM enriched proteins

Gene names	Mean ACM	Mean NCM		Gene names	Mean ACM	Mean NCM		Gene names	Mean ACM	Mean NCM
Cst3	423.6667	56.33333		Igfbp3	29.16667	0.333333		Gm2a	12.66667	0
Apoe	161.1667	14.66667		Col5a2	29	0		Sfrp1	12.5	0
Vim	138.6667	21.33333		Dbi	28.66667	5.333333		Mmp2	12.33333	0
Ncan	131.5	29.33333		Plod1	28.5	0.333333		Akap12	12.33333	0.666667
Serpinf1	122.1667	1.333333		Gpc4	27.5	0.333333		Gpc1	12.16667	4
C3	112.5	3		Pla2g7	27.33333	0.333333		Lgals1	12.16667	1.333333
A2mp	110.3333	1.333333		Vcl	26.5	1		Ctsc	11.83333	0
Sparc	102	5.333333		Hexb	25.83333	0		Ecrg4	11.5	0
Fn1	94	0.666667		Pebp1	25.66667	0		Tnr	11.33333	1.333333
Igfbp2	91	1.666667		Prdx6	25.16667	4.333333		Ptprz1	11.33333	1
Clu	85	4		Spon1	24.83333	1.666667		Pkm	11.16667	1.333333
Igfbp5	84.5	9		Agrn	24	3.666667		Loxl3	11.16667	0
Cdh2	77.5	18.33333		Csf1	23.16667	0		Fmod	11.16667	0
Ctsb	72	5.666667		Serpine2	22	0		Cpq	11.16667	0
Ctsl	69	2.333333		Efemp2	22	0.333333		Islr	11.16667	0
Actn4	68.5	11		Nid2	21.66667	0		Fabp7	10.83333	4
B2m	66.66667	6		Tpm1	21.66667	4.333333		Sema3c	10.83333	2.333333
Ctsd	65.16667	1		Myh9	21.33333	0.666667		Lfng	10.5	0
Timp2	62.66667	19		Plec	21.33333	0		Csf1r	10.5	0.333333
Sparcl1	61.83333	12.33333		Col11a1	20.83333	0		Itm2b	10.33333	2
Msn	55.16667	3.666667		Flnb	20.66667	0		Hsp90b1	10.33333	4.666667
C4b	54.83333	0.333333		Sdf4	20.33333	1		Heg1	10.33333	0
Tagln	54.66667	9		Nes	19.33333	0		Ptgds	10.16667	0
Nucb1	53.16667	7		Htra1	19.16667	0		Pea15	10.16667	0.666667
Grn	51.83333	2		Ezr	18.83333	0		Ctbs	10.16667	0
Ctsz	49.5	1		Pdgfrl	18.66667	0.333333		Tpm4	10.16667	5
Hspg2	49	0		P4hb	18.5	7.333333		B4gat1;B3gnt1	10	0.666667
Fstl1	48.83333	0.333333		Clstn1	18.5	0.333333		Serpinh1	9.833333	0.666667
Lyz2	48.66667	5.666667		Nrcam	17.66667	0		Thbs1	9.833333	1
Lgmn	48.16667	0		Igfbpl1	17.5	0.333333		Galnt2	9.666667	1.666667
Tnc	44.83333	0.333333		Gns	17.33333	3.666667		Col4a1	9.666667	0
Serpina3n	42.16667	0		Gfap	17	0		Lamc1	9.666667	0
Psap	41.83333	3.333333		Bgn	16	0		Tpp1	9.5	0
Bcan	41.33333	0		C1ra	15.66667	0		Sod1	9.5	0
Actn1	41.16667	0		Col1a1	15.66667	0		Ldlr	9.5	0
Ogn	40.66667	0		Cspg4	15.5	0		Eno1	9.333333	0
Man2b1	40.33333	0		Lgals3bp	15.5	0		Glo1	9.166667	2.333333
Vcam1	39.33333	0		Ppt1	15	1.333333		Fam20c	9.166667	0
Pdia3	39.33333	21		Actc1;Acta1	14.5	7.333333		Cdh4	9.166667	0.666667
Ctss	38.5	2		Rnase4	14.33333	0		Col5a1	9.166667	0
Dag1	37.5	1		Tpm4	13.83333	0		Metrn	8.833333	0.333333
Flna	35.5	0		Gpi	13.83333	1		Lama5	8.666667	0
Sod3	33.33333	0		Arsb	13.66667	0.333333		Hexa	8.5	0
Cpe	33	11.33333		Pros1	13.5	0		Ctsd	8.5	0.666667
Ppia	32.83333	0.666667		Mxra8	13.33333	0		Glud1	8.333333	0.333333
Lrp1	32.33333	1		Lmna	13.16667	8.333333		Plod3	8.333333	0
Gstm1	31.83333	0		Qsox1	13.16667	0		Pdia4	8.333333	3.666667
Tf;Gm20425	31.5	0.333333		Scg3	12.83333	7		Galnt16	8.333333	0
Cp	30	0.666667		Gsn	12.66667	0		Igfbp7	8.166667	0
Ctsa	29.83333	0		Txnrd1	12.66667	3		Myl6	8.166667	4.666667

Figure A8 (2/4)

Gene names	Mean ACM	Mean NCM	Gene names	Mean ACM	Mean NCM	Gene names	Mean ACM	Mean NCM
S100a11	8.166667	0	Clic1	5.5	0	Wisp2	3.833333	0
Ctgf	8.166667	0	Col18a1	5.5	0	Got1	3.833333	0
Erp29	8	3	Ggh	5.5	0	Ctso	3.833333	0
Epdr1	8	0	Slc3a2	5.5	0	Cd109	3.833333	0
Olfm2	7.833333	1.333333	Col6a1	5.5	0	Pcdhgc5	3.833333	0
Cd14	7.666667	0	Ppic	5.5	0	Thy1	3.833333	0
Man2b2	7.666667	0	C1qb	5.5	0	Olfml3	3.833333	0
St8sia2	7.666667	0	Vasn	5.5	0	Ifi30	3.666667	0
Tagln2	7.666667	0	Axl	5.333333	0.333333	Ctsf	3.666667	0
Mcam	7.5	0	Itih5	5.333333	0	Serping1	3.666667	0
Slit2	7.333333	0	Prss23	5.333333	0	Sema6d	3.666667	0
Gas1	7.333333	0	Col3a1	5.333333	0	Pls3	3.666667	0
Aldoa;Aldoart2	7.166667	0	Manf	5.166667	2.666667	Dkk3	3.666667	0
Wdr1	7.166667	3.333333	Ly86	5.166667	0	Psma1	3.666667	0
Fabp5	7.166667	0	Asah1	5	0	Col4a5	3.666667	0
Ecm1	7.166667	0	Ext1	5	0	Man1a1	3.666667	0
Calu	7.166667	1.333333	Npc2	5	0	Rnh1	3.666667	0
Pcolce	7	0	Glod4	5	0	Sgsh	3.833333	0
Creg1	7	0.333333	Mfge8	4.833333	0	Wisp2	3.833333	0
Metrnl	7	0	Ak2	4.833333	0.333333	Got1	3.833333	0
Pcdhgc3	7	0	Ext2	4.833333	0	Ctso	3.833333	0
Col1a2	6.833333	0	C1qc	4.833333	0	Cd109	3.833333	0
Bmp1	6.833333	0	Minpp1	4.833333	0	Pcdhgc5	3.833333	0
Pltp	6.833333	0	Cdh11	4.833333	0.333333	Thy1	3.833333	0
H2-Q4	6.833333	0	Akr1a1	4.666667	0.333333	Olfml3	3.833333	0
Celsr2	6.833333	0	Prdx1	4.666667	0	Ifi30	3.666667	0
Galns	6.666667	0	Ctsh	4.666667	0	Ctsf	3.666667	0
Prkcsh	6.666667	2.666667	Plxnb2	4.666667	0	Serping1	3.666667	0
Plbd2	6.5	0	Cnn2	4.666667	0	Sema6d	3.666667	0
Cant1	6.5	0	Tcn2	4.666667	0	Pls3	3.666667	0
Tln1	6.333333	0	Cd44	4.666667	0	Dkk3	3.666667	0
Txndc5	6.333333	0	Pnp;Pnp2	4.666667	0	Psma1	3.666667	0
Ptk7	6.333333	0	Efemp1	4.5	0	Col4a5	3.666667	0
Mgat5	6.333333	0	Gpc6	4.5	0	Man1a1	3.666667	0
Cfh	6.333333	0	Ldha	4.5	0	Rnh1	3.666667	0
Thbs3	6.333333	0	Igfbp4	4.333333	0	Lamb2	3.5	0
Fbn1	6.166667	0	Itih3	4.333333	0.333333	Timp1	3.5	0
Cstb	6.166667	0	Chi3l1	4.333333	0	Nme1	3.5	0
Efna5	6.166667	0	Igf1	4.166667	0	Pdlim5	3.5	0
Plod2	6.166667	0	Nid1	4.166667	0	Prnp	3.5	1
Me1	6	0	Col12a1	4.166667	0	Igsf8	3.5	1
Fbxo2	6	0	Cyr61	4.166667	0	Adamts1	3.5	0
Aga	5.833333	0	Ddr1	4	0	Naglu	3.333333	0
C1sa	5.833333	0	Lbp	4	0	Asrgl1	3.333333	0
Gusb	5.666667	0	Pam	4	0.666667	Dkk3	3.333333	1
Cd81	5.666667	0	Scg5	4	0.666667		3.333333	0
Trem2	5.5	0	Cfp	4	0	Plxdc2	3.333333	0.333333
Megf10	5.5	0	Pgk1	4	0	Anxa3	3.333333	0
Ncl	5.5	0	Rnase1	3.833333	0	Aebp1	3.166667	0
Lrp4	5.5	0.333333	Sgsh	3.833333	0	Arsa	3.166667	0

Figure A8 (3/4)

Gene names	Mean ACM	Mean NCM	Gene names	Mean ACM	Mean NCM	Gene names	Mean ACM	Mean NCM
Tpm2	3.166667	0.333333	Lum	2.166667	0	Lman2	1.666667	0
Fbln2	3.166667	0	Ly86	2.166667	0	Enpp2	1.666667	0
Egfr	3.166667	0	Gm8251	2.166667	0	Pdcd6ip	1.666667	0
Ccl4	3	0	Fgfr1	2.166667	0	Flnc	1.666667	0
Atp6ap1	3	0	Lta4h	2.166667	0	Gas6	1.666667	0
Cfl1	3	0		2.166667	0	Adam12	1.666667	0
Cadm1	3	0	Erp44	2.166667	0.333333	Slit3	1.666667	0
Gnat3	3	0	Xylt1	2.166667	0	Ddt	1.666667	0
Hdhd2	3	0	Rplp1	2.166667	0	Smpd1	1.666667	0
Plau	2.833333	0	Lect1	2.166667	0	Csrp1	1.666667	0
Sema3b	2.833333	0	Shisa5	2.166667	0	Scrn1	1.666667	0
Park7	2.833333	0	Cnn1	2.166667	0	Palld	1.666667	0
Sema3e	2.833333	0	Vegfa	2.166667	0.333333	Pcna	1.5	0
Oaf	2.833333	0	Sorl1	2.166667	0	Ftl1	1.5	0
Fhl1	2.833333	0	B4galt1	2	0.333333	Ahnak	1.5	0.333333
Ifnar2	2.833333	0	Txn	2	0.666667	Mt2	1.5	0.333333
Spp1	2.833333	0	Nov	2	0.333333	Lpl	1.5	0.333333
Pcsk6	2.833333	0	Purb	2	0	Ass1	1.5	0
Sema4b	2.666667	0	Lamp2	2	0	Tbca	1.5	0
Tmem132b	2.666667	0	Pgls	2	0	Dpp7	1.5	0
Hyal1	2.666667	0	Col4a2	2	0	Eda2r	1.5	0
Aplp2	2.666667	0	Cd9	2	0	Anxa2	1.5	0
Aldh1l1	2.666667	0	Mfap4	2	0	Pxdn	1.5	0
Ufm1	2.666667	0	Tpbg	2	0	Fut11	1.5	0
Ltbp2	2.5	0	Gmnc	1.833333	0.333333	Colec12	1.5	0
Tgfb2	2.5	0	Hyou1	1.833333	0	Adam22	1.5	0
Nit2	2.5	1	Chrdl1	1.833333	0	Hspg2	1.5	0
Dld	2.5	0	Cdh6	1.833333	0.333333	Epb41l2	1.5	0
Igdcc4	2.5	0	Sorcs1	1.833333	0		1.5	0
Adam17	2.5	0	H2-L	1.833333	0	Map4	1.5	0
Cnpy2	2.5	0.666667	Dcn	1.833333	0	Tgfbi	1.5	0
Phactr2	2.5	0	Sirpa	1.833333	0	Prdx5	1.5	0
Omd	2.5	0	Qsox2	1.833333	0	Itpa	1.5	0
Cx3cl1	2.5	0	Hepacam	1.833333	0	Lrp8	1.5	0
Lamb1	2.5	0.333333	C1qa	1.833333	0	Rarres1	1.333333	0
Sema3f	2.5	0	Draxin	1.833333	0	Olfm1	1.333333	0
Btd	2.5	0	Ganab	1.833333	0	S100a10	1.333333	0
Taldo1	2.5	0	Hspb1	1.833333	0	Fth1	1.333333	0
Boc	2.5	0	Nrp1	1.833333	0	Igf2	1.333333	0
Sgce	2.333333	0	Rbbp9	1.666667	0	Ptprg	1.333333	0
Pik3ip1	2.333333	0	Ldhb	1.666667	0	Camk2d	1.333333	0
Cxcl16	2.333333	0	Gsta4	1.666667	0	Npnt	1.333333	0
Serpini1	2.333333	0	Chst11	1.666667	0	Vwa5a	1.333333	0
Hs3st1	2.333333	0	Nptn	1.666667	0	Ephb2	1.333333	0
Fgfbp3	2.333333	0	Rcn2	1.666667	0.333333	Prdx4	1.333333	1
Arpc1b	2.333333	0	Flna	1.666667	0	Ephb3	1.333333	0
Mt3	2.166667	1	Sptan1	1.666667	0	Rsu1	1.333333	0
Myl12b	2.166667	0.333333	Cxcl10	1.666667	0	Idh1	1.333333	0
Vcp	2.166667	0	Cryab	1.666667	0	Fkbp1a	1.333333	0
Lcp1	2.166667	0	Hadh	1.666667	0	Acta2	1.333333	0

Figure A8 (4/4)

Gene names	Mean ACM	Mean NCM		Gene names	Mean ACM	Mean NCM		Gene names	Mean ACM	Mean NCM
Slc9a3r1	1.333333	0		Adam15	1.166667	0		Lxn	1	0.333333
Adam9	1.333333	0		Pcmt1	1.166667	0		Crocc	1	0.333333
Myl9	1.333333	0		Galc	1.166667	0		Rpe	1	0
Hmgb2	1.166667	0		Fam198b	1.166667	0		Lrtm2	1	0
Mia	1.166667	0		Arpc5	1.166667	0		Jam2	1	0
37500	1.166667	0		Ywhab	1.166667	0		Npc1	1	0
Smpdl3a	1.166667	0		Scg2	1	0.333333		Got2	1	0
C1rb	1.166667	0		Npvf	1	0.333333		Itgb1	1	0
Golm1	1.166667	0		Ccl3	1	0		Fbln1	1	0
Apeh	1.166667	0.333333		Sptbn1	1	0		Cd84	1	0
Tpm3-rs7	1.166667	0		Manba	1	0		Cd248	1	0
Sema5b	1.166667	0		Lcn2	1	0		Sh3bgrl	1	0
Pdlim1	1.166667	0		Bag3	1	0		Coro1c	1	0
Lamc1	1.166667	0		Ccdc80	1	0		Ndst1	1	0
Ccl2	1.166667	0		Fdps	1	0		Crb2	1	0
Clec3b	1.166667	0		Gpnmb	1	0		Ddah1	1	0
Fkbp3	1.166667	0		Idi1	1	0.333333		Psma6	1	0
Nenf	1.166667	0		Oog3	1	0.333333		Fbln5	1	0
Dner	1.166667	0.333333								

Figure A8: Relative expression of proteins enriched >2-fold in ACM over NCM

Mean ACM is the average of relative protein abundance in 6x mouse ACM samples (3x samples in NBA media, 3x samples in DMEM media – protein expression was comparable between media conditions). Mean NCM is the average of relative protein abundance in 3x rat NCM samples. Proteins detected (with relative level ≥ 1) in ACM with an enrichment >2-fold ACM:NCM are given in the table.

Figure A9

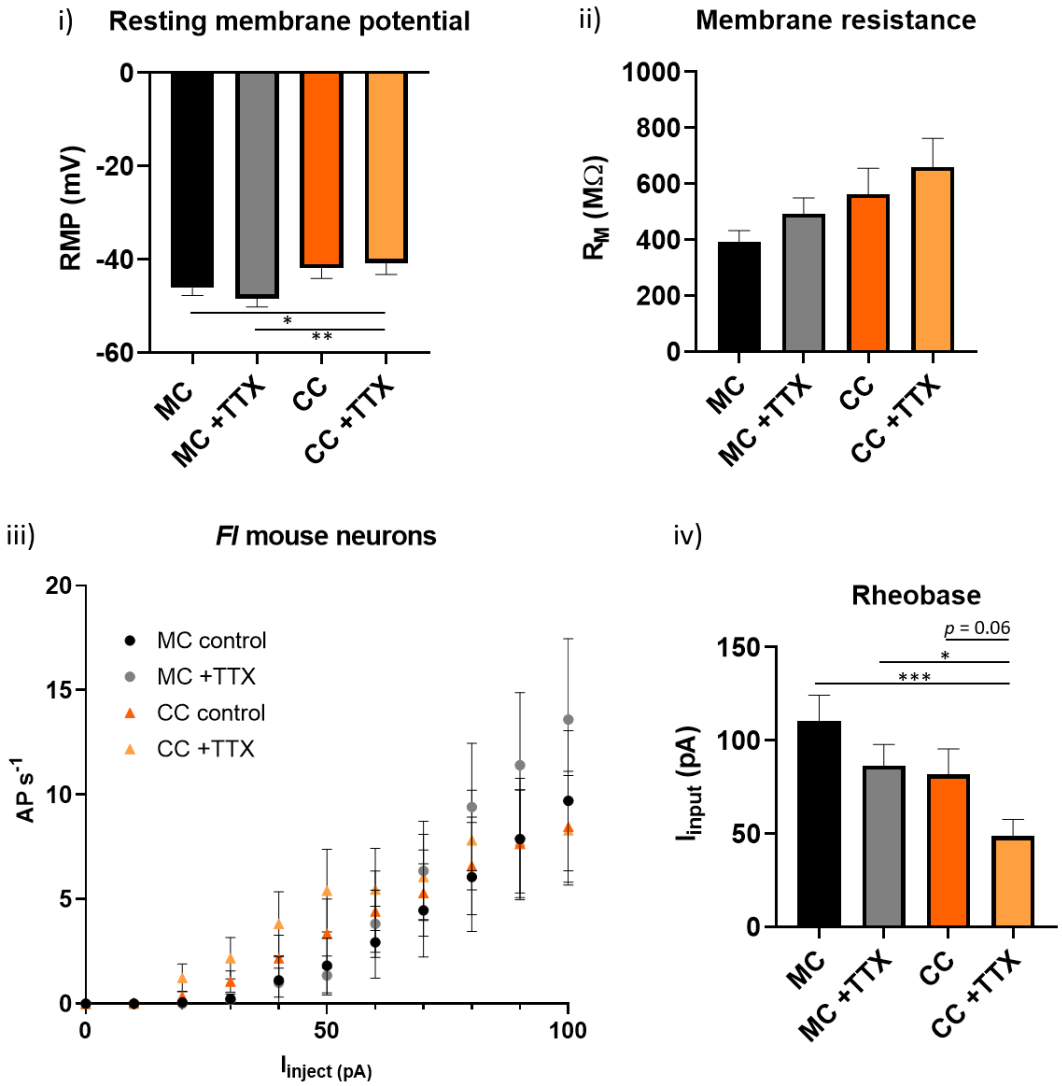


Figure A9: Astrocytes alter the membrane properties of mouse neurons at DIV8, appearing similar to rat neurons at DIV15

Mouse neurons were seeded instead of rat neurons and the intrinsic properties recorded at DIV8 to check for the effects of mixed species. At DIV8 there was no apparent difference between MC and CC mouse neurons. However, treatment with TTX for 48hrs saw an emergence of an effect of astrocytes. i) There was a significant difference between CC +TTX neurons and both MC and MC +TTX neurons ($p = 0.046$ and $p = 0.008$ for MC and MC +TTX, respectively, LME ANOVA, df = 60). ii) CC +TTX neurons had a slight but insignificant increase in RM compared to MC +TTX ($p = 0.02$ and $p = 0.1$, respectively). iii) CC neurons treated with TTX have a slight leftward shift in their FI relationship compared to other conditions. iv) With TTX treatment CC neurons have significantly lower rheobase compared to both MC and MC +TTX neurons, and a slight but insignificant reduction compared to untreated CC ($p < 0.001$, $p = 0.03$, and $p = 0.06$, compared to MC, MC +TTX and CC, respectively).

Figure A9 (accompanying text)

One question arising from the experimental set-up using mixed species (mouse astrocytes and rat neurons) is whether the effects of astrocytes on neurons is due to an effect of different species on gene expression, rather than different cell type. The results of the labs' RNA-sequencing on mixed mouse preparations shown in appendix *Figure A6* suggest that this is not the case, but to address this concern, I created a same species set-up, using mouse cortical neurons grown on mouse astrocytes. I then recorded the membrane properties and excitability in these same species MC and CC cells. As mouse primary neurons typically develop slightly faster than rat neurons, I additionally created samples of MC and CC mouse neurons that had been incubated in TTX for 48 hrs prior to recording. This was to control for any homeostatic masking of differences in properties, as described in the previous section, that may occur at an earlier time point in mouse cells.

At DIV8 there was no significant difference in RMP, R_M, or excitability between untreated MC and CC mouse neurons. However, in TTX-treated cells differences emerged. There was a significant depolarisation in RMP between in CC +TTX treated cells compared to MC +TTX (*Fig A9 i*: mean = -48.4 ± 1.8 and -40.8 ± 2.4 mV, n = 17 and 17, for MC +TTX and CC +TTX, respectively), as well as a slight but insignificant increase in R_M (*Fig A9 ii*: mean = 493.6 ± 56.2 and 659.3 ± 103.4 MΩ for MC +TTX and CC +TTX, respectively, $p = 0.1$). In terms of excitability, activity-deprived CC neurons additionally had a slight but insignificant leftward shift in their *FI* relationship, with significantly lower rheobase than TTX-treated MC mouse neurons (*Fig A9 iii & iv*: mean input current = 86.5 ± 11.3 and 48.8 ± 8.9 pA, n =17 and 17, for MC +TTX and CC +TTX, respectively).

Although there are shifts in developmental stage and speed, it appears that the effects of astrocytes on neuronal properties are not species specific, with mouse neurons also benefiting from an increase in excitability when grown with mouse astrocytes.

Figure A10

	Gene	Mean FPKM <40	Mean FPKM >40	Relative expression >40
Notch downstream genes	Hes1	9.3	6.6	0.7
	Hes6	2.9	1.3	0.4
	Hes5	3.5	0.9	0.3
	Hey2	1.4	1.6	1.1
	Hey1	10.4	9.8	0.9
	Bcl2	5.4	4.7	0.9
	Total FPKM	*32.9*	*24.8*	*0.8*
Notch receptors	Notch2	22.4	14.4	0.6
	Notch1	0.8	0.4	0.5
	Notch3	0.5	0.2	0.4
	Notch4	0.1	0.1	1.2
	Total FPKM	*23.8*	*15.1*	*0.6*
γ-secretase	Psen1	7.9	7.0	0.9
	Psen2	1.2	0.7	0.5
	Ncstn	24.6	11.6	0.5
	Aph1a	1.6	1.3	0.8
	Aph1b	5.1	4.7	0.9
	Psenen	0.3	0.7	2.5
	Total FPKM	*40.6*	*25.9*	*0.6*
Effector/ activators	Maml1	1.8	0.9	0.5
	Med8	4.4	2.8	0.6
	Rbpj	6.5	7.2	1.1
	Furin	0.5	0.2	0.4
	Total FPKM	*13.2*	*11.1*	*0.83623*
	Total Notch related (FPKM)	***110.6***	***76.9***	***0.695315***
Glutamate transporters	Slc1a2	2454.5	1521.3	0.6
	Slc1a3	1146.6	797.8	0.7
	Total FPKM	***3601.0***	***2319.1***	***0.644***

Figure A10: Both Notch pathway and astrocytic EAAT transporter genes are downregulated with age in humans

On average, downstream Notch genes as well as genes for the Notch receptors, γ-secretase complex and Notch effector complex show lower expression in brain samples from humans >40 years old (n = 6) compared to samples from humans <40 years old (n = 6), suggesting that there is a decrease in Notch signalling activity with age in humans. Additionally, a similar decrease in the expression for the astrocytic glutamate transporters *SLC1A2* and *SLC1A3* is likewise observed in humans with age. Data from Zhang et al., 2016.